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Use of micellar electrokinetic capillary chromatography in the study of in vitro metabolism of phenol by human liver microsomes

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Abstract

Micellar electrokinetic capillary chromatography (MECC) is used for analysis of microsome incubation samples. Phenol, a neutral analyte, is separated from interferences. The separation is achieved without sample clean-up. MECC is shown to be advantageous for situations where rapid sampling with minimum handling is desired.

Keywords: Human liver microsomes; Metabolism; Micellar electrokinetic capillary chromatography; Phenol

1. Introduction

The body is exposed to numerous xenobiotics on a daily basis. It has long been established that the cytochrome P_{450} enzymes of the liver play a major role in the body's effort to detoxicate and eliminate foreign compounds. These enzymes catalyse a variety of reactions including oxidations, reductions and dehalogenation. P_{450} enzymes accept a wide range of compounds as substrates. Products of the reactions are generally more hydrophilic than their corresponding substrates, making them more easy to elimate from the body.

Among the oxidation reactions catalysed by P_{450} enzymes is the insertion of a single atom of oxygen from molecular oxygen, resulting in hydroxylation of the substrate. Both aliphatic and aromatic substrates may be hydroxylated in this way. The overall reaction is

$$SH + O_2 + NADPH + H^+ \xrightarrow{P_{450}} SOH + H_2O + NADP^+$$

where SH is the substrate, SOH is the hydroxylated product and NADPH serves as an electron donor. The hepatic cytochrome P_{450} system involves an electron transport pathway that includes NADPH-cytochrome P_{450} reductase, a flavoprotein with both FAD and FMN as prosthetic groups. P_{450} requires its first electron from P_{450} reductase. The second electron may be donated by P_{450} reductase or by NADH-cytochrome b_5 reductase.

Because the hepatic cytochrome P_{450} system has mono-oxygenase activity, it is anticipated that phenol would be oxidised by microsomes to form the hydroxylated product, hydroquinone. This metabolite has been observed in vivo in rat liver using microdialysis sampling [1].

Capillary zone electrophoresis (CZE) separates analytes on the basis of differential migra-

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tion in an electrical field. The technique offers rapid, high resolution separation of components in complex mixtures, with only small quantities of samples required for the analysis. Amino acids, peptides [2] and proteins [3] are amenable to analysis by CZE, as are numerous small molecules [4].

Anions and cations are separated in an electrical field as a result of differences in magnitude of charge and in electrophoretic mobilities. All neutral species are carried by electro-osmotic flow and are not separated from one another. Changing the pH of the running buffer may ionise some species, resulting in separations. This approach is not practical if extreme pHs are required. Micellar electrokinetic capillary chromatography (MECC) is a mode of CZE that uses surfactants for the formation of micelles [5]. In MECC, neutral species are partitioned between the hydrophobic micelles and the hydrophilic buffer. In some cases, further separation is achieved by the additional of organic modifiers to the running buffer [6].

As CZE is suitable for protein analysis, it is possible to analyse microsome incubation mixtures directly. At the physiological pH of 7.4, both phenol and hydroquinone are predominately neutral species, requiring the MECC mode of operation to separate them.

2. Experimental

Phenol and hydroquinone were obtained from Reidel-de Haën. Sodium dodecyl sulphate was obtained from BDH Chemicals and HPLC grade acetonitrile from LabScan. All other chemicals were reagent grade or better and used as received.

Human liver microsomes were prepared by the sucrose method [7] and were obtained from Uwe Fuhr, Department of Clinical Pharmacology, University Hospital, Frankfurt, Germany. Liver microsomes from methylcholantrene induced rats were obtained from IFFA CREDO (France).

Analysis of all samples and standards were performed on a Beckman P/ACE System 2050 CZE with UV detection at 214 nm for all experiments. Automation of the system was achieved by use of an IBM personal computer with System Gold software for instrument control and data collection. The capillary was an unmodified fused silica of 50 μm i.d., 27.2 cm total length and 20.2 cm distance to the detector window. The capillary was maintained at 25 °C.

Various running buffer components, concentrations, pH, and additives were tested in order to separate phenol and hydroquinone from each other, and from substances present in and generated by the microsomes during incubation. A running buffer consisting of about 13 mM phosphate and 7 mM borate with 50 mM SDS and 10% (by volume) acetonitrile was used for all analyses reported here. The separation potential was raised to 15 kV over 0.2 min and held there for the duration of the sample analysis. The separation potential resulted in an operating current of about 50 μA. Prior to each injection, the capillary was rinsed for 1 min with 0.1 M NaOH followed by a 1 min rinse with running buffer. Hydrodynamic injections were made by application of 0.5 psi pressure difference across the capillary for a duration of 3 s.

For each set of human liver microsomes, a blank incubation (no phenol present) and three replicates with phenol were performed. A blank incubation and two replicates with phenol were carried out with rat liver microsomes. The components and their concentrations in the initial incubation mixture are shown in Table 1. The NADPH required by NADPH-cytochrome P_{450} reductase was supplied in the incubation mixture from the action of glucose-6-phosphate-dehydrogenase on glucose-6-phosphate. This enzyme required $NADP^+$ as a hydrogen acceptor, generating NADPH. All components were prepared in 25 mM phosphate buffer (pH 7.4).

Appropriate volumes of each component were placed in 1.5 ml microcentrifuge tubes and mixed. The microsomes were added last and an initial sample (≈ 20 μl) was analysed immediately after addition of the microsomes. Incubation mixtures had an initial volume of

Table 1
Incubation Mixture

Component	Initial concentration
Phenol [a]	30 mg ml^{-1}
Glucose-6-phosphate	2 μg ml^{-1}
Glucose-6-phosphate dehydrogenase	4 units ml^{-1}
$NADP^+$	96 g ml^{-1}
Microsomes	$\approx$2 mg protein ml^{-1}

[a] Phosphate buffer (pH 7.4) was substituted for phenol solution in blank incubations.

250 µl and were maintained at 37 °C during sampling. Subsequent samples were taken at 6.5 min intervals and analysed immediately.

Phenol peaks in the electropherograms were converted to concentrations by means of standard calibration curves which were linear over the range of concentrations in the incubation mixtures. Hydroquinone calibration curves were also linear. For purposes of comparing the incubations of microsomes obtained from various donors, phenol concentrations were normalised on the basis of the initial sample peak in each incubation.

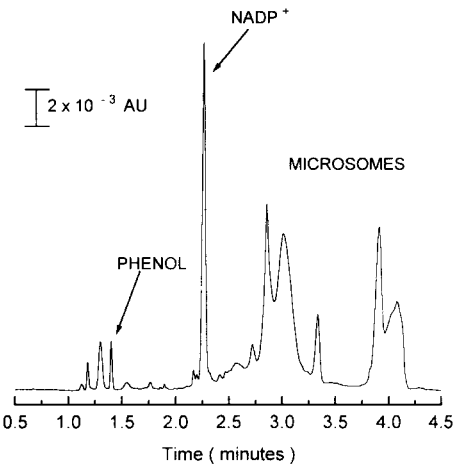

Fig. 2. Electropherogram of incubation mixture.

3. Results and discussion

Fig. 1 shows an electropherogram of a blank incubation sample with one of a phenol and hydroquinone standard superimposed. Peak A, present in both electropherograms, is due to differences between the composition and pH of the running buffer and the buffer in which the injections were prepared. Analysis of the series of samples for incubations showed two early migrating peaks produced by microsome activity, even in the absence of substrate. The peaks, B and C in the blank, increased in intensity with time during the incubations. It can be seen that the hydroquinone peak, B, in the standard injection, nearly co-migrates with the first of the microsome peaks. In some incubation samples, the microsome peaks and hydroquinone produced from phenol appeared as two very small fused peaks but could not be reliably quantified. Peak D, the peak due to phenol, is clearly separated form the peaks produced by microsome activity. A typical electropherogram for an incubation sample is shown in Fig. 2. The phenol and $NADP^+$ peaks have been identified. The microsomes migrated in two broad groups. NADPH co-migrated in the earlier of these groups.

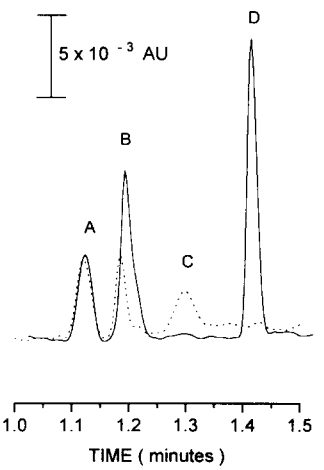

Fig. 1. Superimposed electropherograms of blank incubation mixture (– – –) and standard containing phenol and hydroquinone (——).

The disappearance of phenol in the incubations was used to follow the metabolic activity of the microsomes. Human liver microsomes from four different donors were tested. Microsomes from donors P20, P13 and P14 clearly metabolised phenol. Donor P18 microsomes did not significantly metabolise phenol. Rat liver microsomes were also tested and showed significant metabolism of phenol. Significance was determined at the 0.05 level by performing the Student's t-test on the first samples compared to the final samples for each donor. Fig. 3 shows phenol concentration versus incubation time for the three human donor microsomes which metabolised phenol and for the rat microsomes. For purposes of comparison, the data have been normalised based on the initial peak. Each point for the microsomes of human donors represents the average of three incubations. Points for rat microsomes are the averages of two incubations. The lines are lines of best fit for the normaslied data. Error bars are the standard deviations for normalised values.

Microsomes from three human donor and the rat microsomes showed significant decreases in phenol over the course of the incubations. The relative decrease by these donors ranged from 8 to 12% after 20 min. After 40 min the relative decreases ranged from 10 to

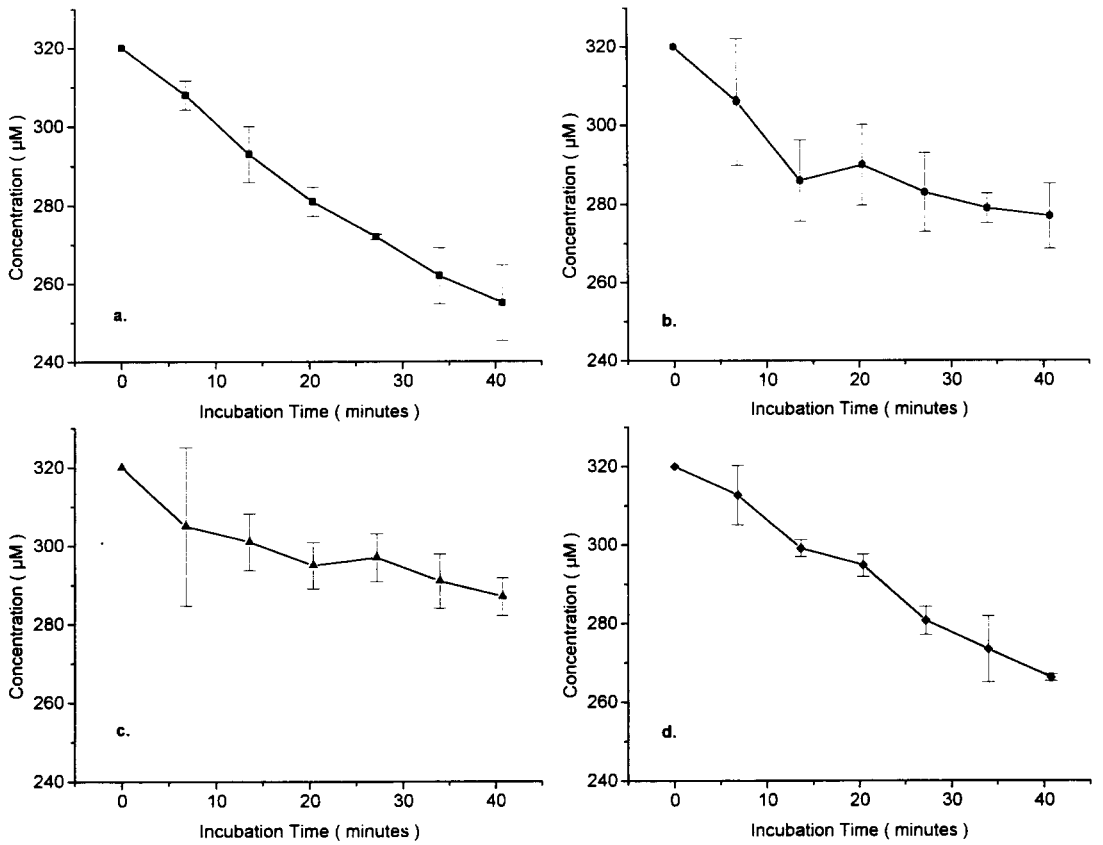

Fig. 3. Changes in concentration of phenol during incubation: (a) donor P20 (■); (b) donor P13 (●); (c) donor P14 (▲); (d) rat (◆).

20%. After 1 h the range of decreases were from 15 to 28%. Microsomes from the fourth donor showed no significant decrease over 60 min incubation. Incubation of phenol in the absence of microsomes did not show a significant change in concentration.

Differences in the extent of metabolism of a substrate by microsomes are not unexpected, as variations in metabolic rates and patterns between species and among individuals within a species have been observed [8]. The differences among humans are thought to be due to genetic factors, deficient levels of some enzymes or differences in environment [9]. Various isoforms of cytochrome P_{450}, including those responsible for mono-oxygenase activities such as aromatic hydroxylation, can be induced [10].

The microsome incubation mixture used in these experiments provided only one pathway for the metabolism of phenol, i.e. conversion to hydroquinone. In the absence of other pathways, more extensive conversion might be expected. The low levels of conversion to hydroquinone observed suggest that phenol may be a poor substrate for the particular P_{450} isozymes present in these donors, or that the isozymes responsible for aromatic hydroxylation are absent or present only at low levels in these donors. The number of donors tested is too small, and the available information insufficient, to define the underlying cause of the low levels of phenol metabolism observed here.

The ability of CZE and MECC to handle direct injection of microsomal incubations samples greatly simplifies the performance of experiments such as these. High-performance liquid chromatography (HPLC) has been widely used for samples analysis in microsome metabolism studies. HPLC generally requires protein-free samples for injection. In the case of microsomal studies, this involves addition of acid or an organic modifier to precipitate the proteins followed by centrifugation. In some cases extraction steps are included for further clean-up and analyte concentration. Each additional step introduces errors and increases the time required for completion of the experiment. The MECC method used in this study required less than 2 min of analysis time for each sample and essentially no sample preparation time.

Samples were removed from the incubation mixture within 60 s of injection into the capillary. Reactions were not quenched prior to injection, as it seemed unlikely that metabolic processes could continue in an electrical field of the magnitude generated by the separation potential. Protein adsorption onto the capillary was not observed in this study, possibly owing to the thorough rinsing of the capillary between injections.

HPLC generally requires longer analysis times than does MECC. When information on later-migrating species is not desired, rinsing the capillary in preparation for the next sample can begin immediately after detectioin of analytes of interest. Any sample remaining in the capillary is flushed out and does not interfere with the next sample. In contrast, HPLC requires either long analysis times or gradient elution to clear later-eluting peaks from the column. When gradient elution is used, the column must be returned to the initial mobile phase conditions, again requiring additional time between sample injections. The short analysis times for MECC coupled with the option to quickly clear the capillary for subsequent samples would be advantageous in experiments where high temporal resolution is required or when labile substances are involved.

The reliability of quantitative data from CZE and MECC methods is dependent on the reproducibility of the injections and the consistency of the operating conditions. Drifts of migration times of less than 6 s during instrument operation times of up to 8 h were observed. An injection of standard phenol solution was made before and after each series of incubation samples to ensure reliable peak identification. To test the reproductibility of injections a series of 15 standard injections were made. The relative standard deviation (RSD) for phenol migration time was 0.8%. Peak heights for the injections had RDSs of 0.9%, while the value for peak areas was 2.4%.

The work reported here demonstrates the usefulness of MECC for analysis of samples containing microsomes. Rapid analysis is possible owing to the separation technique and because sample clean-up is not necessary. Instrumentation which offers automated sample injection and temperature control of the capillary provides reproducible injections and consistent operating conditions. Automation facilitates regimes for rinsing and thus reconditioning the capillary to speed sample throughput and to minimise protein adsorption onto the capillary.

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