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Short communication

Determination of tamoxifen and metabolites in serum by capillary electrophoresis using a nonaqueous buffer system

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Abstract

Tamoxifen (TAM), an antiestrogen, is widely used to treat hormone-dependent breast cancer in post-menopausal women. TAM may be used as a chemopreventive agent in women of child-bearing age; however, few data exist describing potential TAM-induced fetal toxicity. In support of the National Toxicology Program's characterization of reproductive and developmental effects of TAM, this work describes an analytical technique utilizing capillary electrophoresis (CE) for the detection of circulating levels of TAM, *N*-desmethyltamoxifen (DMT), and 4-hydroxytamoxifen (4-HT) in maternal rodent serum. Greater than 90% of ^{3}H-labeled TAM was extractable from serum using 98:2 hexane–isoamyl alcohol. Optimum separation of TAM, DMT, and 4-HT was obtained on a 57 cm×50 μm capillary using a nonaqueous buffer system of 1:1 methanol–acetonitrile containing 50 m*M* ammonium acetate and 1% acetic acid. 4-Dimethylaminopyridine was used as internal standard. Temperature and voltage were optimized at 40°C and 15 kV, respectively. The limit of detection of TAM by UV detection at 214 nm was approximately 800 amol. TAM and DMT were confirmed in serum of female rats 4 h following a single oral dose of 120 mg/kg. Transplacental exposure of TAM to fetal tissue will be evaluated using this technique.

Keywords: Tamoxifen; *N*-Desmethyltamoxifen; 4-Hydroxytamoxifen

1. Introduction

The use of oral contraceptives has contributed to a decline in the incidence rates of ovarian and endometrial cancers over the past few decades [1]. Unfortunately, contraceptive use has shown no concomitant chemopreventive activity toward breast cancer. The antiestrogen, tamoxifen (TAM) has been effective for treatment of estrogen-responsive tumors associated with metastatic breast cancer [2], therefore TAM is currently being evaluated as a possible chemopreventive agent against breast cancer [1,3]. However, prolonged prophylactic TAM administration to women of child-bearing age is of special concern. Although TAM appears to be relatively acutely non-toxic [2], there is some evidence that the risk of endometrial cancer increases in women undergoing long-term therapeutic TAM administration [3]. Furthermore, TAM has been shown to be a hepatic carcinogen in rats, apparently associated with the formation of hepatic DNA-adducts following metabolic activation of the parent compound [4,5]. Two TAM-derived metabolites, *N*-desmethyltamoxifen (DMT) and 4-hydroxytamoxifen [4-HT] have been detected in serum of rats, mice, and human cancer patients following oral administration of TAM [6]. Additionally, DMT, several hydroxylated products including 4-HT, and at least two epoxides have been detected in either rodent and/or human liver microsomal incubates [7]. Both α- and

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4-HT have been postulated as genotoxic derivatives of TAM associated with liver carcinogenesis [8–10] and a peroxidase-activated epoxide intermediate may be involved in carcinogenic activity at extrahepatic sites [11].

Presently, the National Toxicology Program (NTP) is characterizing reproductive and developmental effects of TAM in rodents as part of the risk-benefit evaluation for use of TAM as a chemopreventive agent in humans. Transplacental exposure of TAM will be determined by analyzing fetal tissue and maternal rodent serum for the presence of TAM, DMT, and a potential proximate metabolite, 4-HT. Currently, the analytical method of choice for detecting TAM-derived material in tissue is HPLC, with detection limits of about 0.2–2 ng total material injected [6]. Recently, Ng et al. [12] described a novel CE-based analytical method for the separation of TAM and several metabolite standards in solution using a nonaqueous buffer system. Advantages of using CE over HPLC are the potential for increased sensitivity, shorter analysis times and smaller sample size. Greater assay sensitivity would be highly advantageous for analysis of TAM-derived material in biological matrices containing very low levels of analyte, as might be expected in fetal tissue following doses of lower concentrations of TAM to dams. Therefore, the present work describes a modified CE analytical technique, based on the use of a nonaqueous buffer system similar to that described by Ng et al. [12] for the separation of TAM, DMT, and 4-HT in serum. The CE system will be used in our laboratory for determining the presence of these compounds in fetal tissue following transplacental exposure to TAM.

2. Experimental

2.1. Extraction of TAM, DMT, and 4-HT from serum

TAM, DMT, and 4-HT were extracted from serum (rat or fetal bovine) using a method similar to that described by Robinson et al. [6]. The procedure involved washing 1 part serum with 5 parts of a 98:2 (v/v) mixture of hexane–isoamyl alcohol in a corex glass tube, vortexing each sample for 0.5–1 min, then centrifuging the sample at approximately 500 *g* for 5 min. Following centrifugation, the aqueous layer was frozen by a dry ice:acetone bath and the organic layer containing TAM, DMT, and 4-HT was poured into a glass conical-bottom vial, placed in a SpeedVac Concentrator (Savant, Farmingdale, NY, USA) and evaporated to dryness. The extraction procedure was repeated twice more, with total recovery of TAM from serum determined to be 92±2%. This value was obtained by spiking serum with [*n*-methyl-^{3}H]TAM (Amersham, Arlington Heights, IL, USA), extracting the radioactivity with hexane–isoamyl alcohol as described, then counting each organic layer directly in scintillation fluid in a Beckman Instruments (Fullerton, CA, USA) model 9800 scintillation counter.

2.2. CE apparatus

Separations of TAM, DMT, and 4-HT were carried out on a Beckman P/ACE 5000 capillary electrophoresis system utilizing a 57 cm×50 μm fused-silica capillary. Detection was by on-capillary UV absorbance measurement at 214 nm. Data were integrated on an IBM Value Point personal computer (Armonk, NY, USA) using Beckman System Gold software.

2.3. Sample analysis

TAM-derived material present in sample vials following solvent evaporation was resuspended in 25–50 μl of 1:1 methanol–acetonitrile containing 10 m*M* ammonium acetate and 1% acetic acid. The internal standard, 4-dimethylaminopyridine (DMAP), was contained in the resuspension buffer. The capillary was washed daily for 10 min with 0.1 *M* NaOH and 5 min with de-ionized H_2O prior to sample analysis. Additionally, a capillary rinse with electrophoresis buffer was carried out for 5 min prior to sample injection. All capillary washes were performed at a pressure of 137.9 kPa. Samples were pressure injected at 3.45 kPa for up to 10 s. Loading capacity and resolution of samples were optimized by utilizing a 30-s voltage (2 kV) injection of 1:1 methanol–acetonitrile containing 10 m*M* ammonium acetate and 1% acetic acid immediately following sample injection. Electrophoretic separation was

performed using a nonaqueous buffer system composed of 1:1 methanol–acetonitrile containing 50 m*M* ammonium acetate and 1% acetic acid. The CE system was operated at a temperature of 40°C and voltage of 15 kV, resulting in a cross-capillary current of approximately 15 μA.

2.4. Calibration curve

Aliquots (20 μl) from serial dilutions originating from two separate solutions of methanol containing TAM, were spiked into 1 ml serum samples, extracted and evaporated to dryness as previously described, resuspended in the CE injection buffer containing a known amount of internal standard, then injected (5 s) and run on the CE system. A calibration curve was generated from the resulting electropherograms based on the ratio of the peak areas of TAM to DMAP. A standard curve for 3–4 replicates at each data point (10, 20, 30, 40, 60, 80, and 100 pg/injection) was constructed and goodness-of-fit was determined by linear regression.

2.5. Animals and treatments

Four female Fischer 344 rats with an average weight of 114 g were dosed by gavage with 120 mg TAM/kg. All rats were sacrificed 4 h post-dosing by CO_2 asphyxiation. Serum was prepared from blood collected by cardiac puncture at the time of euthanasia.

3. Results and discussion

This work describes a CE-based analytical method for detecting circulating levels of TAM and a major metabolite, DMT in serum (see structures, Fig. 1). Previously, TAM and its metabolites have been characterized in biological tissues and fluids using HPLC [6,13]. Using CE as an alternative analytical method to HPLC has the potential to increase sensitivity of the assay, an important issue when dealing with low levels of TAM-derived material that might be expected in fetal tissues following administration of clinical doses to dams. Ng et al. [12] have previously reported on a CE method utilizing a nonaqueous buffer system that separated

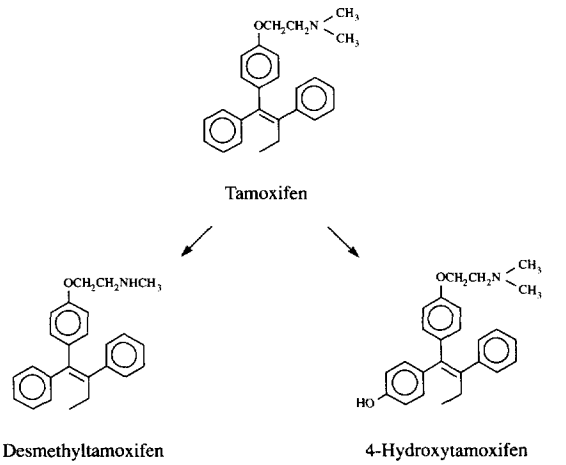

Fig. 1. Structures of the test compounds undergoing chromatographic separation in the CE system.

standards of TAM and several metabolites contained in solution. We have modified that method to successfully extract and separate TAM, DMT, and 4-HT from spiked serum and to confirm the presence of TAM and DMT in serum of TAM-treated rats.

Data from the present CE method was tested for linearity and levels of detection of TAM. The method was linear at $r^2=0.981$ with a slope and intercept of 0.006 ± 0.013 and 0.009 ± 0.0002, respectively. Standard error of the linear fit was 0.031. The experimental LOQ was 10 pg total injected; the LOD, based on a signal to noise ratio of 3, was determined to be approximately 800 amol (data not shown). Sensitivity of the method was improved by the use of the UV absorbance detector at 214 nm compared to the more widely used 254 nm wavelength.

Separation of TAM from DMT and 4-HT was greatly influenced by capillary temperature as shown in Fig. 2. TAM and DMT essentially co-migrated at ambient temperature. Resolution improved as the temperature was increased, with baseline separation of TAM and DMT standards achieved at 35°C. Optimum resolution was reached at 40°C for TAM and DMT extracted from a biological matrix (Fig. 3). The 4-HT standard existed as isomeric forms (mainly trans), with near baseline separation of the isomers achieved in the CE system. Isotachophoresis preconcentration (previously described for a nonaqueous buffer system in an investigation of

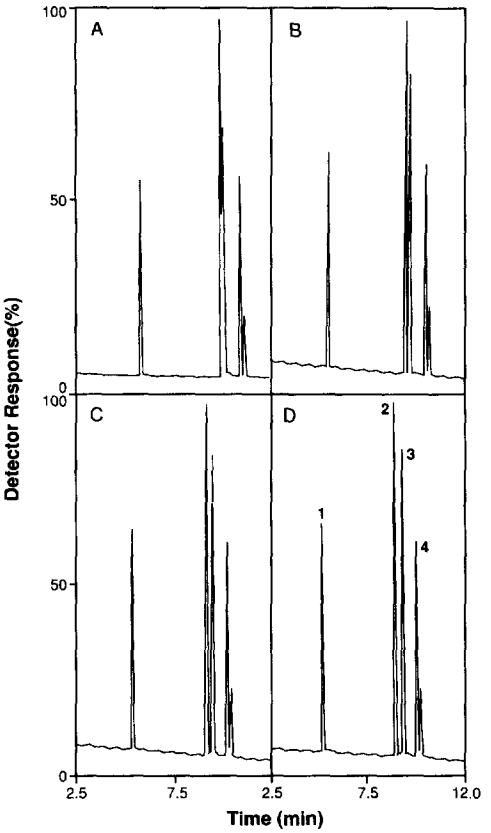

Fig. 2. Typical CE electropherograms showing the effect of temperature on the separation of (1) DMAP, (2) DMT, (3) TAM, and (4) 4-HT (exists as isomeric forms). Panels A, B, C, and D represent electropherograms of standards run on the CE system at temperatures of 20, 25, 30, and 35°C, respectively.

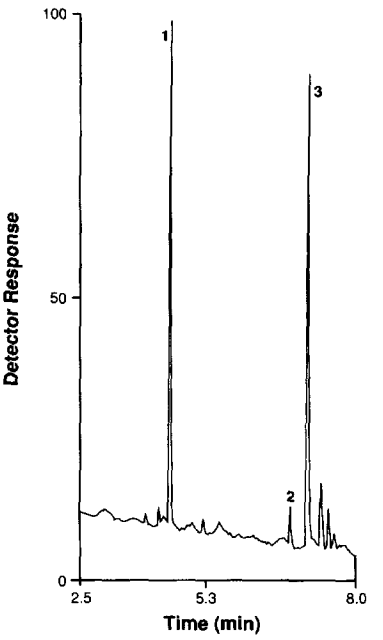

Fig. 3. Typical CE electropherogram of a serum extract sample derived from a rat treated with 120 mg TAM/kg by gavage. Solvent extraction and CE analysis of TAM-derived material was carried out as described in Section 2. Peak 1 is the internal standard (DMAP). Peaks 2 and 3 are DMT and TAM, respectively (see Fig. 4).

pyrazoloacridine metabolism in vivo [14]) was used to enhance resolution of the test compounds and to increase the loading capacity of the capillary. In our CE system, 10 s was the optimum injection time, since injection times of over 10 s resulted in peak broadening with no appreciable increase in peak height (data not shown).

A serum extract sample from a TAM-treated rat was serially spiked with standards of TAM, DMT, and 4-HT (Fig. 4). Both TAM and DMT clearly co-migrated with corresponding peaks intrinsic to the sample. Evidence indicated that 4-HT was also present in serum of TAM-treated rats, however, confirmation was complicated by the fact that 4-HT can exist as isomeric forms and up to three peaks may be present in the area of 4-HT migration (Fig. 4). The peak migrating immediately following the parent TAM peak in serum extract clearly was not 4-HT. This as yet unidentified peak may be another hydroxylated TAM-derived metabolite such as α-hydroxytamoxifen.

4. Conclusion

A CE system has been described that can be used to quantitate TAM and a major metabolite DMT in serum. The advantage of this system over HPLC analysis of TAM-derived material in biological fluids and tissues is the increased sensitivity of the assay. Since detection limits were in the amol range for TAM, it is our goal to utilize this CE-based analytical method for the detection of low levels of TAM-derived material in rodent fetuses following transplacental exposure to the compound and/or its

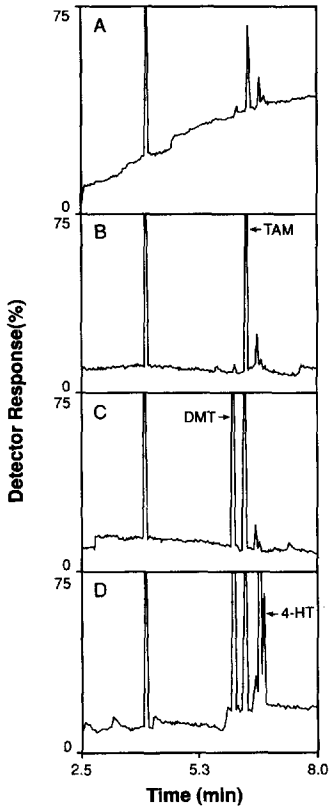

Fig. 4. Electropherogram A represents a serum extract sample derived from a TAM-treated rat. Electropherograms B, C, and D, represent the serial addition to sample A of TAM, DMT, and 4-HT, respectively.

metabolites. Future work in our laboratory will be designed to address this issue.

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