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Note

Determination of polycyclic aromatic amines in skin by liquid chromatography with electrochemical detection

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The development of synthetic fuels has raised concerns over the health hazards associated with coal derived liquids. Recent health effects studies have shown that some coal liquids contain quantities of genotoxic and carcinogenic polycyclic aromatic amines[1–3]. The potency of many of these aromatic amines has given impetus to studies aimed at understanding their mode of action. Of particular interest is the disposition of polycyclic aromatic amines following dermal exposure because that is considered to be the most likely route of exposure for synfuels workers. This report describes methodology developed in our laboratory for the analysis of several polycyclic aromatic amines in rat and mouse skin using liquid chromatography with electrochemical detection (LC–ED). Although the methodology was developed for measurements of skin absorption rates, it should be adaptable to the analysis of aromatic amines in other tissues.

There are a number of recent reports in the literature on the analysis of aromatic amines in coal liquids in which thin layer, column or liquid chromatography were used for sample clean-up in preparation for analysis by gas chromatography or gas chromatography–mass spectrometry[4–6]. Polyaromatic amines in synthetic fuel mixtures have also been analyzed directly by LC using peroxyoxalate chemiluminescence detection[7]. However, little attention has been given to the analysis of this compound class in biological matrices, although Shaikh *et al.*[8] have measured 2-aminoanthracene and its metabolites after *in vitro* incubations with rat liver S9 fractions by LC after conversion to methylurea derivatives.

The analysis of biological tissues and fluids generally requires methods that are highly selective as well as sensitive. Since the primary aromatic amines are readily oxidized at the carbon electrode, LC–ED offers an attractive approach to their analysis. The sensitivity and selectivity of the LC–ED technique is well documented and has resulted in analytical methods for a variety of other compound types in complex biological matrices. Several reports have illustrated the potential of LC–ED for the analysis of aromatic amines. The detection of picomole quantities of naphthylamines has been demonstrated by Mefford *et al.*[9]. Caudill *et al.*[10] reported on the ability of

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the electrochemical detector to discriminate between the aromatic amine, 2-aminoanthracene, and the corresponding aromatic hydrocarbon, anthracene. The response of the electrochemical detector to a number of one- and two-ring aromatic amines has been examined by Concialini *et al.*[11]. Our report illustrates the utility of the LC–ED technique for the analysis of polycyclic aromatic amines in rodent skin samples. The method presented here also provides a basis for development of procedures for analysis of polycyclic aromatic amines in body fluids and other tissue types.

EXPERIMENTAL

Materials

Aromatic amines, with the exception of 6-aminochrysene, were purchased from Ultra Scientific Corporation (Hope, RI, U.S.A.). 6-Aminochrysene was purchased from Aldrich (Milwaukee, WI, U.S.A.). All solvents were Burdick & Jackson (Muskegon, MI, U.S.A.) distilled-in-glass. Citric acid and trisodium citrate were Baker (Phillipsburg, NJ, U.S.A.) reagent grade and sodium perchlorate was obtained from MCB (Gibbstown, NJ, U.S.A.). All chemicals were used as received.

Apparatus

The liquid chromatograph consisted of a Waters 6000A pump, a Waters WISP 710B automatic sampler, a Kratos Spectroflow 773 variable-wavelength ultraviolet detector and a Bioanalytical Systems Model LC-4A electrochemical detector. Chromatographic peaks were integrated using a Hewlett-Packard 3390A integrator. The electrochemical detector was equipped with a glassy carbon thin-layer electrode referenced to a Ag/AgCl electrode.

High-performance liquid chromatography

Separations were performed on a DuPont Zorbax ODS 25 cm × 4.6 mm I.D. column operated at room temperature (23°C). DuPont C_8 and Waters CN columns were also evaluated during method development. The mobile phase was composed of acetonitrile–citric acid buffer. The buffer was prepared from equimolar quantities of citric acid and trisodium citrate (total citrate concentration from 2.5 m*M* to 5.0 m*M*) and sodium perchlorate (0.1 *M*) in water. For the analysis of skin samples, the acetonitrile–buffer ratio was 70:30. The retention volume for 6-aminochrysene was 12.20 ml at a solvent flow-rate of 1 ml/min. A 20–100-μl volume of tissue extract or of a standard prepared in acetonitrile was injected for each analysis.

Skin painting and extraction

Studies were conducted using Sprague-Dawley CD rats and CD-1 mice (Charles River, Portage, MI, U.S.A.). For extraction efficiency studies, the animals were shaved, killed with carbon dioxide, and skin samples excised and placed on ice immediately. Mouse skin samples were approximately 2 cm in diameter and rat skin approximately 4 cm. Aromatic amines were added to the skin samples by application of either 25 μl or 250 μl of an acetone solution containing approximately 40 μg/ml of each of the amines to be tested. The solution was applied evenly to the external surface of the skin sample using a disposable glass-tipped micropipet. Mouse skin

samples were extracted twice by sonicating for 10 min with 2-ml aliquots of acetonitrile, followed by a final rinse with 1 ml of acetonitrile. The acetonitrile portions were combined in a 5-ml volumetric flask, diluted to volume with acetonitrile, and analyzed without further treatment. Rat skin samples were handled in the same manner, except that two 10-ml aliquots of acetonitrile were used for the extraction and a 5-ml aliquot for the rinse, due to the larger size of the samples. Extracts from rat skin were diluted to a final volume of 25 ml and analyzed without further treatment.

The dermal absorption of 6-aminochrysene in mice was studied by applying 9.00 μg of 6-aminochrysene in acetone to the shaved backs of live aminals. The study in rats was performed in the same manner, except that 9.85 μg was used. Animals were sacrificed with carbon dioxide at selected intervals after dosing, the area of application identified under an ultraviolet lamp, and the skin excised immediately. Skin samples were taken 0, 1, 4 and 24 h after treatment for rats, and at 0, 0.25, 0.5, 1, 4, and 24 h for mice. Excised skin samples were placed in the first extraction volume of acetonitrile (2 ml for mice, 10 ml for rats) in a vial and stored refrigerated overnight before continuing with the extraction and analysis. The extraction and analysis were performed in the same manner as for the recovery studies.

RESULTS AND DISCUSSION

After examining a number of reversed-phase chromatographic systems (C_8, C_{18} and CN), we found that a C_8 or C_{18} column with a mobile phase composed of 40–70% acetonitrile in a aqueous citric acid buffer provides a flexible system for analyzing aromatic amines. The chromatography can be optimized for specific amines by adjusting the percentage of acetonitrile and the pH of the citrate buffer. The effect of acetonitrile content on the capacity factors of several aromatic amines is shown in Table I. Reducing the pH of the mobile phase by substituting citric acid for the citric acid–trisodium citrate buffer caused a dramatic reduction in the retention of the amines. Sodium perchlorate was incorporated in the solvent as a supporting electrolyte for the electrochemical detection. The separation of several aromatic amine standards is shown in Fig. 1.

The selectivity of the electrochemical detector for aryl amines relative to N-

TABLE I

CAPACITY FACTORS (k') OF SELECTED AROMATIC AMINES AS A FUNCTION OF THE ACETONITRILE–CITRATE BUFFER RATIO IN THE MOBILE PHASE

Column: DuPont Zorbax ODS 25 cm × 4.6 mm. Buffer: 5 m*M* citrate (2.5 m*M* citric acid, and 2.5 m*M* trisodium citrate), 0.1 *M* sodium perchlorate. $k' = (t_R - t_m)/t_m$ where t_R is the elution time for the analyte and t_m is the elution time for the solvent (or an unretained solute).

Compound	*Percentage acetonitrile*		
	45	*55*	*65*
2-Aminonaphthalene	5.7	3.6	2.2
4-Aminobiphenyl	9.1	5.1	2.8
2-Aminoanthracene	15.5	7.8	3.9
6-Aminochrysene	36.2	15.7	6.6

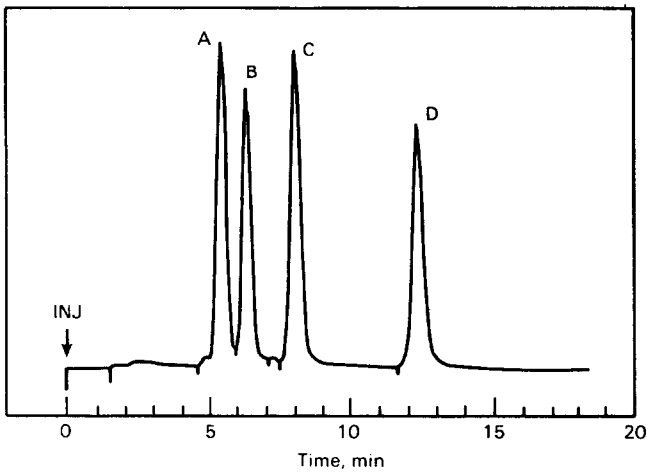

Fig. 1. Separation of aromatic amine standards with acetonitrille. 5 m*M* citrate (65:35). Peaks: A = 2-aminoaphthalene, 43 ng; B = 4-aminobiphenyl, 38 ng; C = 2-aminoanthracene, 41 ng; D = 6-aminochrysene, 39 ng. Chromatographic conditions as described in the text.

heterocyclic compounds was tested by chromatographing several compound types and simultaneously monitoring the electrochemical signal and the ultraviolet absorption at 254 nm. Compounds chosen were representative of the types commonly encountered in synfuels. The results are summarized in Table II. As was anticipated, the aromatic heterocyclic nitrogen compounds were not readily oxidized at the glassy carbon electrode and were not electrochemically detected even at concentrations several orders of magnitude greater than the minimum detectable concentrations of the primary aromatic amines. Carbazole was the only heterocyclic nitrogen compound tested that gave a response. However, it differs from the other heterocycles in being a secondary amine with the nitrogen external to the aromatic system rather than a part of it.

Although carbazole was detected electrochemically, it is possible to detect the

TABLE II

ELECTROCHEMICAL DETECTOR RESPONSE TO AROMATIC NITROGEN COMPOUNDS

ND = None detected. Conditions: C_{18} column with 1 ml/min acetonitrile–5 m*M* citrate (45:55) and glassy carbon electrode at +0.9 V *versus* an Ag/AgCl reference electrode.

Compound	*Nanomoles injected*	*Area/mole*
Quinoline	3.11	ND
4-Azafluorene	1.26	ND
Benzo[*c*]cinnoline	1.27	ND
5,6-Benzoquinoline	1.18	ND
Carbazole	0.392	0.0745
4-Aminobiphenyl	0.0437	4.41
2-Aminonaphthalene	0.0552	6.20
2-Aminoanthracene	0.0823	4.01
6-Aminochrysene	0.168	3.28

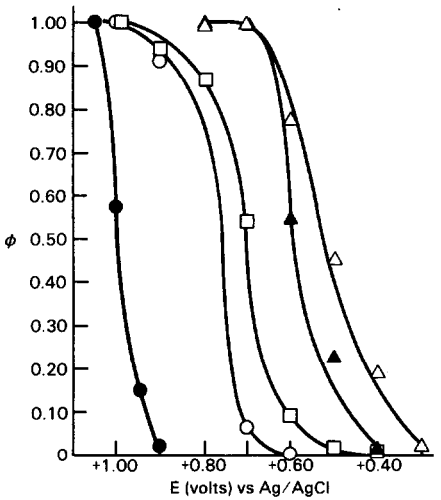

Fig. 2. Hydrodynamic voltammograms of several aromatic nitrogen compounds. ϕ (= the ratio of the peak current to the limiting current) for carbazole was calculated as the ratio of the peak current to the current at +1.05 V. Compounds: ● = carbazole; ○ = 4-aminobiphenyl; □ = 2-aminonaphthalene; △ = 2-aminoanthracene; ▲ = 6-aminochrysene.

aromatic amines in its presence without interference by appropriate selection of the working electrode potential. Some selectivity among the different aryl amines can also be achieved by careful selection of the working electrode potential. The relationship between detector response and voltage for several aryl amines is shown in the hydrodynamic voltammograms in Fig. 2. Adjustment of the electrode potential to +0.7 V, for example will allow detection of 6-aminochrysene without interference from carbazole, which is much more difficult to oxidize. Compounds that have lower oxidation potentials will be detected, while those that have much higher potentials will not.

The electrochemical detector response for three of the aromatic amines was examined in detail and found to be linear over the range of 10 pmoles to at least 500 pmoles injected. Linear regression parameters for several of the compounds are given in Table III. Deviations from detector linearity with injections of 1 nmole or more are typical of many compounds analyzed by LC–ED. If the analytical system were optimized for sensitivity, it would not be unrealistic to expect a minimum detection limit of approximately 0.1 picomole for the more easily oxidized amines. However,

TABLE III

LINEAR REGRESSION PARAMETERS

y = Area response; x = moles of amine.

Compound	*Regression line*	*Correlation coefficient*
2-Aminonaphthalene	$y = 2.22x \cdot 10^6 + 1.35 \cdot 10^4$	0.998
2-Aminoanthracene	$y = 3.30x \cdot 10^6 + 4.04 \cdot 10^3$	0.995
6-Aminochrysene	$y = 2.77x \cdot 10^6 + 5.88 \cdot 10^3$	0.999

TABLE IV

RECOVERY OF SELECTED AROMATIC AMINES FROM SPIKED SKIN

Compound	*Recovery (%)**		
	Mouse		*Rat*
	1 μg	*10 μg*	*10 μg*
2-Aminonaphthalene	98.5– 99.5	99.2– 99.6	101.2–102.0
2-Aminobiphenyl	104.1–107.1	98.9–101.7	103.0–105.1
2-Aminoanthracene	99.5–104.0	96.8– 98.7	97.9– 98.6

* The range of recoveries is given for 3 replicates.

a significant effort would be required to achieve such high sensitivity, and analytical precision would be reduced.

The LC–ED method has been used to monitor the disappearance of 6-ami-

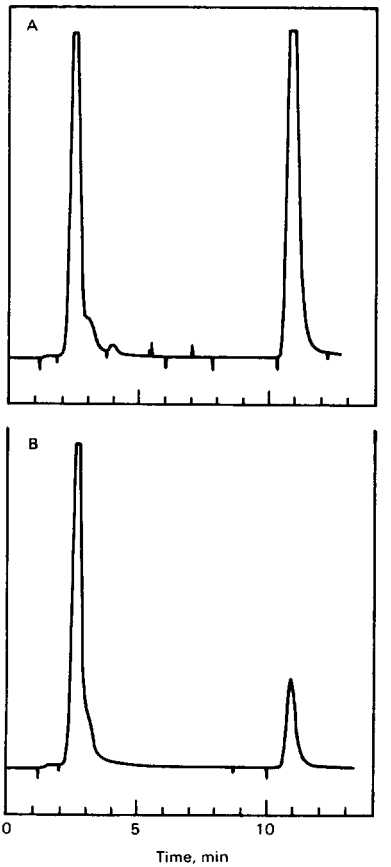

Fig. 3. Chromatograms of mouse skin tissue extracts after application of 9.0 μg 6-aminochrysene at (A) 0 h and (B) 4 h.

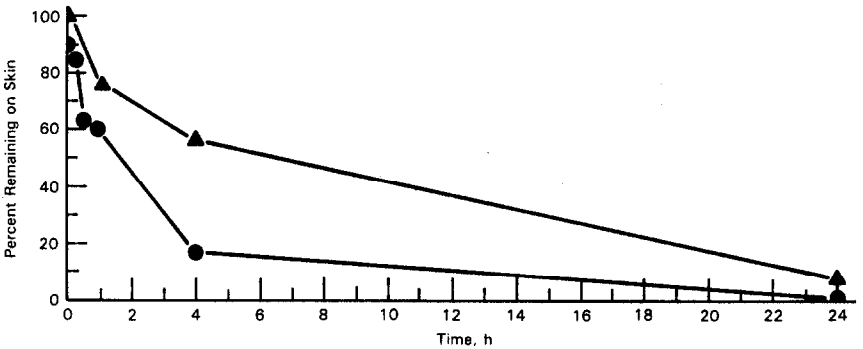

Fig. 4. Percentage of 6-aminochrysene remaining in skin *vs.* time after skin painting. ▲ = Rat, ● = mouse.

nochrysene from skin following topical application to rats and mice. Extraction of the skin with acetonitrile gave nearly quantitative recoveries for all the primary aromatic amines studied, as shown in Table IV. The recovery of 6-aminochrysene from rat skin was 98.9% ± 3.6 (S.D.), $n = 6$ at the 1-μg level and 97.4% ± 3.7 (S.D.), $n = 6$ at the 10-μg level. Chromatograms of the tissue extracts were very clean, as shown in Fig. 3, and permitted the quantification of at least 40 pmoles of a primary aromatic applied to the skin. Increased sensitivity could be gained by concentration of the acetonitrile; however, sensitivity was not a limiting factor in our studies and the effect of sample concentration was not explored. Fig. 4 shows representative data obtained by LC–ED for the skin painting of 6-aminochrysene in both the rat and mouse and illustrates the species variation for skin clearance.

The LC–ED approach provides a rapid, sensitive and selective method for analysis of primary polycyclic aromatic amines in epidermal tissue. In addition, the methodology presented may serve as a basis for the analysis of aromatic amines in other tissues and fluids.

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