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Note

Dihydrocaffeic acid: a common contaminant in the liquid chromatographic—electrochemical measurement of plasma catecholamines in man

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High-performance liquid chromatography with electrochemical detection (HPLC—ED) after an alumina extraction provides high sensitivtiy and specificity for measuring plasma levels of the endogenous catecholamines norepinephrine (NE), epinephrine (E), and dopamine (DA) [1]. This technique will also detect catechols other than the neurotransmitter catecholamines, because the alumina separation and electrochemical reaction depend on a catechol nucleus and its oxidation potential and not on the presence of an amine residue [2]. This

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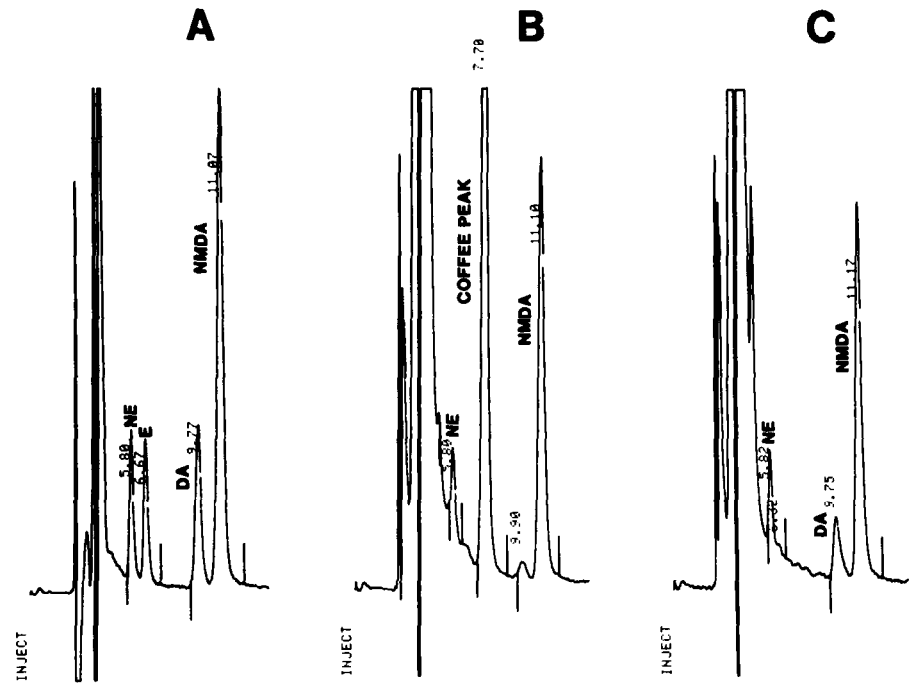

Fig. 1. (A) Chromatogram (BAS system) of 500 pg each of norepinephrine (NE), epinephrine (E), dopamine (DA), and the internal standard, N-methyldopamine (NMDA) after alumina batch extraction. Numbers are retention times in hundredths of a minute. (B) Chromatogram of plasma-derived eluate from a patient with a large coffee peak. (C) Chromatogram from the same patient 18 h after discontinuing coffee. Note complete absence of the coffee peak.

means that in clinical settings where the food and drug intake of the patient is uncontrolled, exogenous catechols may produce contaminating chromatographic peaks by the HPLC—ED technique.

One unidentified substance which we have frequently encountered has been more than a mere annoyance, because it may co-chromatograph with E or DA depending on the chromatographic conditions. In the case shown in Figs. 1 and 2, for instance, this unknown substance co-chromatographed with E in a patient with an adrenal mass and suggested the diagnosis of pheochromocytoma. We sought to identify this substance.

METHODS

We compared the chromatographic characteristics of the unknown substance with those of several standards on different chromatographic systems. The small amount of substance in the unknown peak precluded direct identification by gas chromatography—mass spectrometry (GC—MS) without substantial concentration and isolation efforts.

The HPLC—ED method used was previously validated in this laboratory [1]. In the present study we used two different HPLC—ED systems. One consisted of a Waters 6000A solvent delivery system, U6K injector, μBondapak

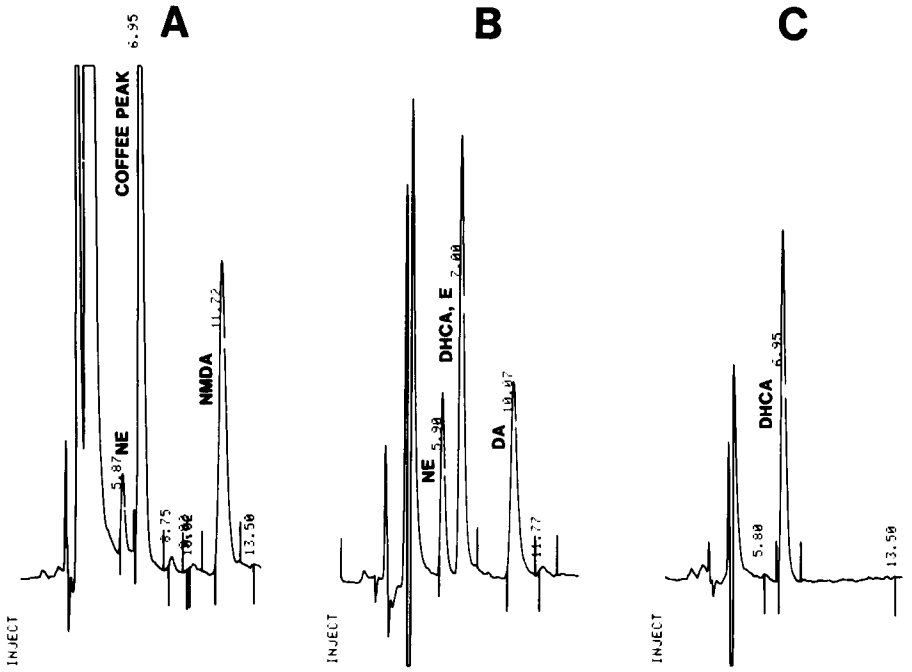

Fig. 2. (A) Co-chromatography of the coffee peak with epinephrine (E) using the same plasma sample as in Fig. 1B but with the plasma-derived eluate injected into a matched system with a somewhat newer column; (B) Co-chromatography of dihydrocaffeic acid (DHCA) with E on this system. Compare with Fig. 1A; (C) Chromatogram of DHCA. Note identical retention time with that of the coffee peak.

C_{18}, 30 cm × 3.9 mm reversed-phase stainless-steel column containing 10-μm irregular particulate packing, and a guard column packed with C_{18} Porasil; a Bioanalytical Systems (BAS) LC4 or LC4A amperometric detector with TL5 glassy carbon electrode; and a Waters Data Module. The mobile phase for this system consisted of 1 l water, 6.8 g sodium acetate, 100 mg EDTA, 1 g heptanesulfonic acid, and 7% acetonitrile at pH 4.8. The mobile phase was pumped at a flow-rate of 1.0 ml/min with the detector set at 1 nA/V at 0.50 V applied potential. The other system was the same, except that an Environmental Sciences Associates (ESA) triple-electrode system was used, with the first electrode in the post-column series set at 0.30 V, the second at 0.15 V, and the third at —0.35 V, so that only reversibly oxidizable species were detected. The ESA mobile phase consisted of 1 l water, 6.8 g sodium acetate, 100 mg EDTA, 1 g heptanesulfonic acid, and 8% acetonitrile at pH 3.5.

After HPLC studies suggested dihydrocaffeic acid as the unknown substance, GC—MS was used to confirm this identification. Aliquots (2 ml) of plasma obtained from subjects without the unknown peak, plasma to which dihydrocaffeic acid (3 ng/ml) had been added, and plasma from a patient who, had a large unknown chromatographic peak were acidified with 1 ml of 1 mol/l hydrochloric acid, and 0.1 ml of 0.114 mol/l ascorbic acid and 0.1 ml of 0.054 mol/l EDTA were added. Each sample was extracted twice with 4 ml of ethyl acetate. The extracts were dried over sodium sulfate, evaporated in vacuo, and transferred with methanol to PTFE-lined screw cap glass tubes. The

samples were evaporated under nitrogen and reacted with 50 μl of pentafluoropropanol and 100 μl of pentafluoropropionic anhydride at 70°C for 15 min, evaporated under nitrogen, and then rereacted with 100 μl of pentafluoropropionic anhydride for 10 min at 70°C. The reacted samples were evaporated to dryness and redissolved in 25 μl of ethyl acetate for GC—MS. A 3% OV-17 (100—120 mesh) column (2 m × 2 mm I.D., glass) was used for analysis. Of each derivatized sample 2—4 μl were injected into a Finnigan Model 3200 electron ionization GC—MS system with a Nermag SADR data acquisition system.

RESULTS

Of 108 people whose plasma was assayed for catecholamine content using HPLC—ED, 36 (33%) had an additional peak larger than that of NE, E, or DA and with a retention time between that of E and DA.

When patients with large unknown peaks were interviewed, they all reported being coffee drinkers, with coffee intake varying from 3 to 30 cups per day. Four subjects volunteered to stop drinking all coffee or caffeinated beverages for periods varying from 18 h to 1 week. In all of these subjects, the unknown peak decreased markedly in height or disappeared entirely (Fig. 1C). Two subjects substituted decaffeinated for caffeinated coffee and had blood drawn

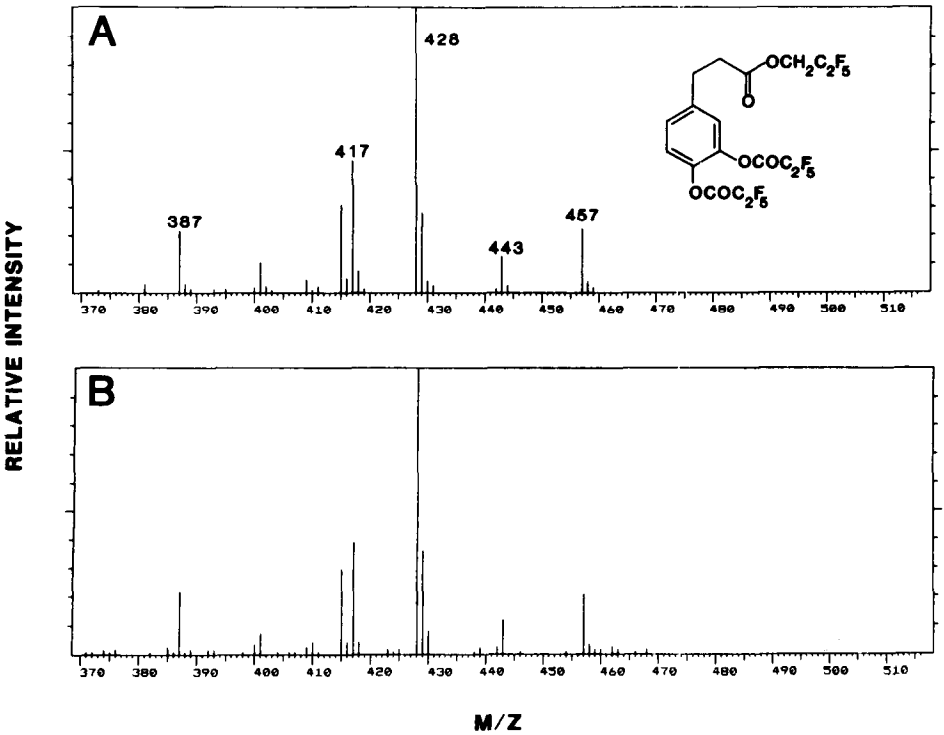

Fig. 3. Electron-ionization spectra for derivatized (A) dihydrocaffeic acid; and (B) plasma extract from a patient with a large unknown peak on HPLC—ED analysis.

1 day or 1 week later, and in both, the unknown peak persisted. These findings, and the fact that neither caffeine nor any of several of its metabolites was detectable by the HPLC—ED procedure, implied that the unknown substance was related to a non-caffeine constituent of coffee. The unknown substance was not detectable in coffee itself after the alumina extraction. Because the unknown substance was retained on alkaline alumina and eluted from acid alumina as part of the HPLC—ED sample preparation, and because it was reversibly oxidizable at low potentials, we reasoned that it was a catechol. None of the known stable catecholamine metabolites or tyramine, synephrine, ascorbic acid, N-methylepinephrine, or dimethylephinephrine co-chromatographed with the unknown substance. We therefore searched for a metabolite of a catechol present in coffee.

Caffeic acid and chlorogenic acid are well known coffee-associated catechols [3]. Caffeic acid was not retained on the HPLC column, and chlorogenic acid did not co-chromatograph with the unknown substance. However, dihydrocaffeic acid (DHCA, 3,4-dihydroxyphenylpropionic acid), a metabolite of caffeic acid, co-chromatographed with the unknown peak on two HPLC systems. DHCA had congruent voltammetry with that of the unknown peak. We therefore concluded that DHCA was likely to be the unknown substance.

Dihydrocaffeic acid was then studied using GC—MS. At a column temperature of 130° C, derivatized DHCA eluted at 2.5 min; the separator and transfer lines were at 200° C; and the ionizing potential was set at 70 eV. Partial mass spectra (*m/z* 370—515) are shown in Fig. 3. Selected-ion recording at *m/z* 428 and 457 (not shown) indicated that these ions were present at the appropriate retention time in the plasma extract which exhibited the unknown peak on HPLC and in the plasma extract to which 3 ng/ml of DHCA had been added but were absent in a plasma sample which did not exhibit the unknown peak on HPLC. Thus, extracted, derivatized plasma without the unknown peak did not contain DHCA, whereas a plasma extract from a subject with a large unknown peak contained a substance with the identical retention characteristics and mass spectra as recorded from authentic DHCA (Fig. 3).

DISCUSSION

In clinical settings where the food and drug intake of the patient is uncontrolled, exogenous catechols may produce contaminating chromatographic peaks by the HPLC—ED technique for catecholamines. One such peak has appeared in about one third of the chromatograms we have obtained, and occasionally this peak has co-chromatographed with epinephrine or dopamine.

On the basis of clinical observations and then HPLC—ED and GC—MS analyses, we identified the unknown substance as dihydrocaffeic acid, a catechol metabolite of caffeic acid. Dihydrocaffeic acid can be produced from caffeic acid by human intestinal bacteria [4].

The presence of detectable DHCA in plasma was not associated with elevations of plasma NE. Thus, despite the fact that acute caffeine ingestion increases plasma catecholamines [5], our apparent marker of coffee drinking was not associated with generalized sympathetic neural activation. When injected intravenously acutely into anesthetized rats and for up to 32 h into

conscious rats, DHCA produced no changes in blood pressure, heart rate, or cardiac output.

We routinely have proscribed drinking caffeinated beverages for 24 h prior to blood sampling in our clinical studies, but we have allowed subjects to drink decaffeinated coffee. Since many subjects have stopped driking coffee in any from prior to blood sampling, the prevalence of the DHCA peak we observed probably underestimated that in the general population.

When HPLC—ED procedures are used to measure plasma catecholamines in people, dietary factors can produce contaminating peaks and potentially lead to erroneous clinical interpretations. One such factor is DHCA, a metabolite of a coffee-containing catechol.

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