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Optimization of high-performance liquid chromatographic assay for catecholamines

Determination of optimal mobile phase composition and elimination of species-dependent differences in extraction recovery of 3,4-dihydroxybenzylamine

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ABSTRACT

This paper describes the application of a window diagram technique to optimize the four components of eluent (sodium acetate, sodium heptanesulfonate, acetonitrile and pH adjusted by monochloroacetic acid), for complete separation of five catecholamine compounds and the internal standard (3,4-dihydroxybenzylamine, DHBA). In addition, studies were performed to address the problem of the variable recovery of DHBA from dog plasma due to a time-dependent loss of DHBA. We found that this phenomenon can be prevented by pH adjustment prior to addition of DHBA, allowing development of an accurate high-performance liquid chromatographic assay for plasma catecholamines in dogs.

INTRODUCTION

Since the publication of results by Felice and Felice [1], reversed-phase ion-pair liquid chromatography (RP-IPC) with electrochemical detection has been widely used for determination of catecholamine concentrations in biological fluids. However, the assay has two deficiencies. Firstly, the C_{18} columns used differ in their performance characteristics even when obtained from a single source, and the characteristics of any individual column vary with use over a period of time. Thus, it is necessary to employ a systematic optimization strategy allowing alteration of mobile phase composition to match the changing column characteristics and consistently provide adequate separation of catecholamines. Secondly, application of the assay to dogs, cattle, horses, sheep and goats can result in erroneously high estimates of catecholamine levels as recoveries of 3,4-dihydroxybenzylamine (DHBA; used as internal standard) in these species have been much lower than those of endogenous catecholamines [2–5]. The present paper addresses these problems in order to develop a reliable assay for catecholamines in dog plasma.

EXPERIMENTAL

Reagents

High-performance liquid chromatography (HPLC)-grade sodium heptanesulfonate (SHS), sodium acetate and analytical-grade disodium ethylenediaminetetraacetate (EDTA) were obtained from Fisher (Springfield, NJ, USA), monochloroacetic acid, L-DOPA, norepine-

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phrine hydrochloride (NE), epinephrine bitartrate (E), dopamine hydrochloride (DA), 3,4-dihydroxybenzylamine hydrochloride (DHBA) and 3,4-dihydroxyphenylacetic acid (DOPAC) from Sigma (St. Louis, MO, USA) and acid-washed aluminium oxide (AAO) from Bioanalytical Systems (West Lafayette, IN, USA).

Chromatography

The chromatographic apparatus consisted of a Waters 6000A pump, a Bioanalytical Systems LC-4B electrochemical detector with TL-8A glass carbon working electrode and RE-C Ag/AgCl reference electrode (Bioanalytical systems), a Waters 740 data module and μBondapak C_{18} column (300 mm × 3.9 mm I.D., 10 μm particle size).

For the procedure to optimize mobile phase composition, two mobile phase solutions were prepared. Eluent A contained no organic phase and consisted of an aqueous buffer with 0.1% (w/v) sodium acetate, 0.02% EDTA, 0.01% SHS with pH adjusted to 2.85 with monochloroacetic acid. Eluent B was composed of 4.0% acetonitrile and 96.0% buffer, the latter containing 0.30% sodium acetate, 0.02% EDTA, 0.10% SHS and monochloroacetic acid to adjust pH to 4.5. A standard solution containing a mixture of DHBA (as internal standard), L-DOPA, NE, E, DOPAC and DA (in 0.2 *M* acetic acid, and stored at 4°C) was then assayed using mixtures of A and B in various ratios (0:100, 25:75, 50:50, 75:25, 100:0) at a flow-rate of 1.0 ml/min, with the detector set at 0.6 V and a sensitivity of 2 nAFS. This allowed determination of retention times for each of the six compounds under five different mobile phase conditions. The resulting data were then used to construct window diagrams as previously described [6–9].

Sample preparation

Blood samples were collected in CAT-A-KIT tubes (Amersham) containing ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) and reduced glutathione. Plasma was separated and stored at −20°C. To 1 ml of thawed plasma were added 50 μl of 5 n*M* sodium metabisulfite, 10 μl of DHBA (400 pg for dog plasma, 900 pg for human plasma), 400 μl of 2 *M* Tris–2% EDTA buffer (pH 8.8) and 10 mg of AAO. The mixture was then shaken for 10 min, centrifuged (2 s in a microcentrifuge) and the supernatant discarded. The AAO was washed three times with 1 ml of deionized water, catecholamines were desorbed with 100 μl of 0.2 *M* acetic acid and the acidic solution was transferred to another tube. A 95-μl volume was injected onto the LC column. In the experiments to optimize DHBA recovery from dog plasma, the order of addition of DHBA and buffer was varied, as was the time period between the additions.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic record of the standard soluton using eluent A (a) and eluent B (b). With eluent A, DOPAC and DA were coeluted and the peaks of L-DOPA and E were poorly separated. With eluent B, DOPAC and DA were well separated, but L-DOPA co-eluted with the solvent front and DOPAC, NE, E and DHBA peaks were close together. The standard solution was further analyzed using mixtures of eluents A and B in the ratios 75:25, 50:50 and 25:75, allowing determination of retention times for each compound under a variety of eluent conditions in which pH and concentrations of sodium acetate, acetonitrile and SHS were simultaneously varied. A plot of these data (Fig. 2) enabled us to determine the parameters t_{R_2}/t_{R_1} [8] and $(t_{R_2} - t_{R_1})/(t_{R_1} + t_{R_2})$ [9] for each of the solute pairs. The resulting window diagrams

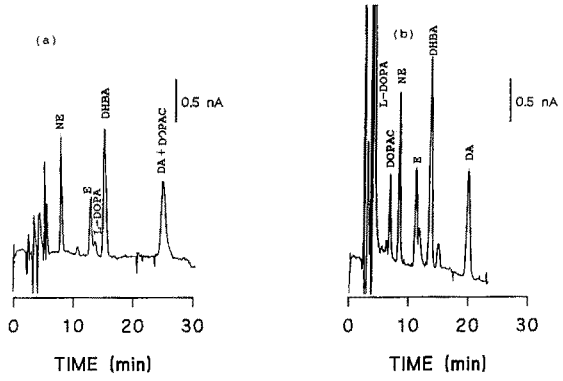

Fig. 1. Chromatographic recordings of a standard solution (L-DOPA 112.9 pg, DOPAC 175.6 pg, NE 230.4 pg, E 260.8 pg, DA 600 pg and DHBA 464 pg) using (a) eluent A and (b) eluent B.

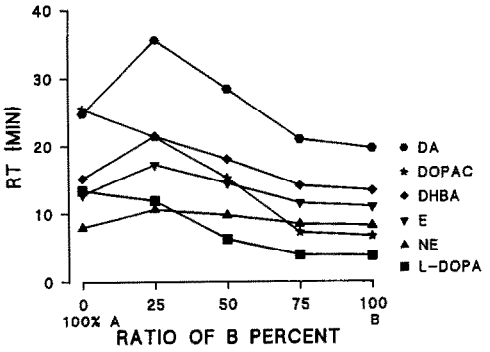

Fig. 2. Relationship of retention time to eluent composition for DHBA and the catecholamines. (Eluent composition is represented as the ratio of admixture of eluents A and B.)

are shown in Fig. 3. Each version gives the same result with three windows of equal height at A/B ratios of 82.7:17.3, 37.7:62.3 and 5.0:95.0.

Thus, there are three possible compositions for the mobile phase that provide optimal separation of all six compounds in aqueous solution: eluent 1 consists of 0.7% acetonitrile and 99.3% buffer containing 0.14% sodium acetate, 0.02% EDTA, 0.0255% SHS and monochloroacetic acid to bring the pH to 3.0; eluent 2 consists of 2.5% acetonitrile and 97.5% buffer containing 0.23% sodium acetate, 0.02% EDTA, 0.066% SHS and monochloroacetic acid to produce a pH of 3.9; eluent 3 consists of 3.8% acetonitrile and 96.2% buffer containing 0.29% sodium acetate, 0.02% EDTA, 0.096% SHS and monochloroacetic acid for a pH of 4.5.

These three optimized eluent solutions were then used in the analysis of the standard solution, human plasma, and human plasma plus standard solution. The resulting chromatograms are illustrated in Figs. 4–6. With eluents 2 and 3 L-DOPA coeluted with the solvent front in both plasma and standard solution (Figs. 5 and 6). Furthermore, the E peak in plasma was obscured by an unknown peak when eluents 2 or 3 were used, as was the DOPAC peak with eluent 2. Eluent 1 was thus determined as the optimal mobile phase composition for catecholamine determination in human plasma.

t_{R_2}/t_{R_1} and $(t_{R_2} - t_{R_1})/(t_{R_1} + t_{R_2})$ were found to be equally effective criteria for the optimization procedure (Fig. 3). In contrast with the criterion of α as developed by Laub and Purnell [10], these criteria do not require measurement of V_0 (volume of elution of the solvent front). This is an advantage because measurement of V_0 can be difficult in HPLC and because the use of α can result in the wrong criterion for optimization when the solute peaks are very close to the solvent front [9].

Improvement of the extraction recovery of DHBA from dog plasma

When, during sample preparation, DHBA was added prior to addition of the Tris–EDTA buffer, calculated NE recoveries were inappropriately high in dog plasma compared with human plasma (249.6+18.9 *versus* 135.4±7%). A series of experiments in which the time period between addition of DHBA and subsequent addition of Tris–EDTA buffer was progressively lengthened showed that the problem resulted from a time-

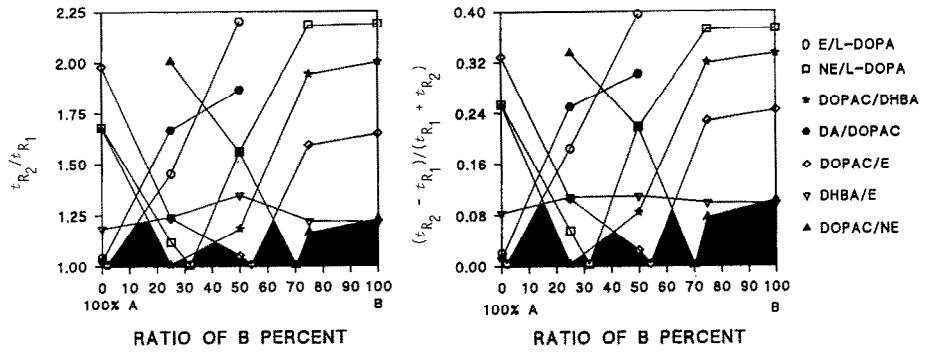

Fig. 3. Window diagrams constructed using relative retention time (t_{R_2}/t_{R_1}) or $(t_{R_2} - t_{R_1})/(t_{R_2} + t_{R_1})$ for each of the solute pairs. In each case there are three windows of equal height occurring with eluent compositions (A-to-B ratios) of 82.7:17.3, 37.7:62.3 and 5.0:95.0. Solute pairs which do not contribute to the windows have been omitted.

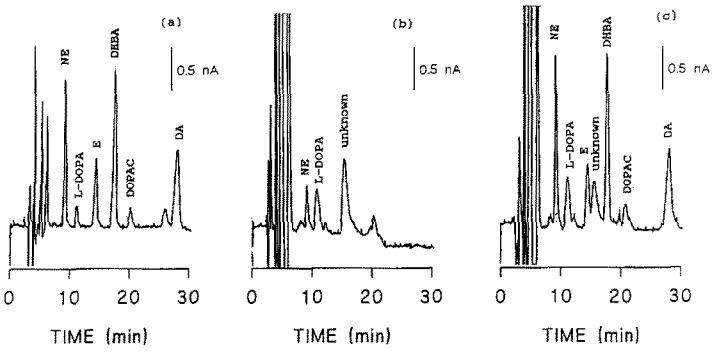

Fig. 4. Chromatographic recordings of (a) standard solution, (b) plasma and (c) plasma plus standard solution, using optimization eluent 1.

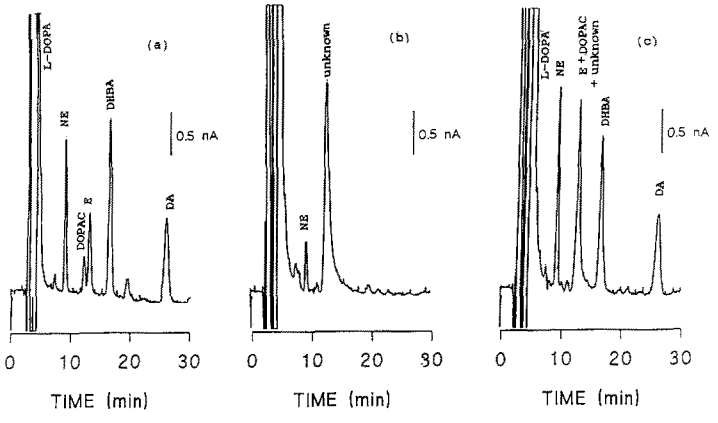

Fig. 5. Chromatographic recordings of (a) standard solution, (b) plasma and (c) plasma plus standard solution, using optimization eluent 2.

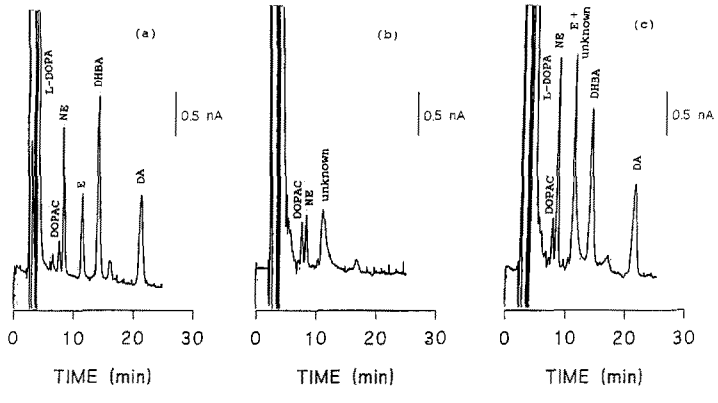

Fig. 6. Chromatographic recordings of (a) standard solution, (b) plasma and (c) plasma plus standard solution, using optimization eluent 3.

dependent loss of DHBA in dog plasma (Fig. 7a and c). These results support those of a previous report [3] that attributed the loss of DHBA to irreversible protein binding. We found the time course of loss of DHBA plateaued after about 40 min suggesting that the process is saturable.

A number of approaches to solving this problem have previously been suggested including the use of [^{3}H]NE as internal standard [2], the addition of Tris–EDTA buffer to each sample tube immediately after addition of DHBA [3] and deproteinization of plasma before addition of DHBA [5]. Each of these methods raises new problems. Thus, [^{3}H]NE was unsuitable as an internal standard for our purposes as we needed to assay [^{3}H]NE in our samples, deproteinization proved time-consuming when large numbers of samples were being prepared, and the method of Robie and DuSapin [3] requires that the interval between addition of DHBA and buffer be identical for each sample (a requirement which is difficult to satisfy). We therefore investigated the possibility of adding the DHBA after addition of the Tris–EDTA buffer (*i.e.*, after adjusting the plasma pH to 8.8). The results of this experiment are shown in Fig. 7b and d). They reveal that the peak-height ratio (NE peak height/DHBA peak height) when DHBA is added after Tris–EDTA buffer is independent of the time interval between additions for both dog and human plasma.

Modifying our technique according to the above findings, our detection limits for NE and E in dog plasma were 10.8 and 12.1 pg/ml respectively. The intra- and interday coefficients of variation of NE and E in human plasma were 7.8 and 7.6% for NE and 9.4 and 10.1% for E and in dog plasma were 5.1 and 7.5% for NE and 11.8 and 12.9% for E.

CONCLUSIONS

The window diagram technique is a valuable tool for selection of components of the mobile phase of RP-IPC. Following empirical determination of the initial mobile phase compositions, window diagram construction allows efficient, simultaneous optimization of multiple components of the mobile phase. Furthermore, the use of t_{R_2}/t_{R_1} or $(t_{R_2} - t_{R_1})/(t_{R_1} + t_{R_2})$ was effective, avoiding the necessity to measure V_0. Finally, the species-dependent difference in extraction recovery of DHBA from dog plasma and human plasma was eliminated by adjustment of the plasma pH to 8.8 before addition of DHBA.

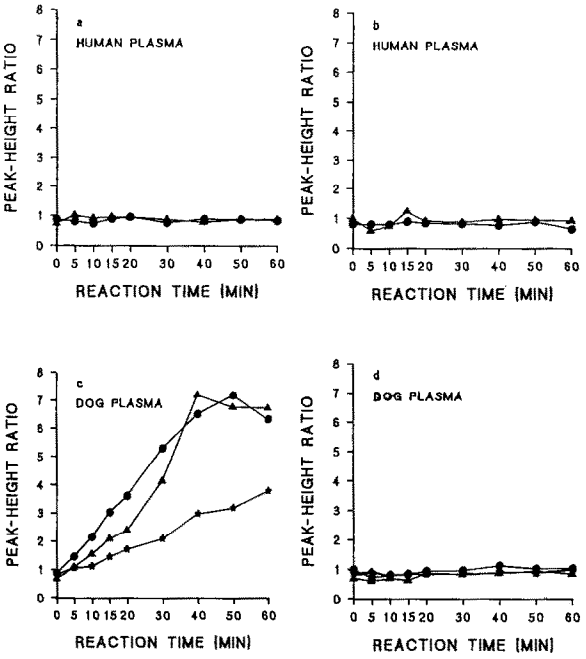

Fig. 7. Effects of varying the time interval between addition of DHBA and addition of buffer on NE/DHBA peak-height ratio. Experiments were performed with human (a, b) or dog (c, d) plasma to which standard solution was added. In a and c, DHBA was added before buffer, in b and d, DHBA was added after buffer. (The peak-height ratios are corrected for the NE present in the plasma prior to addition of standards.)

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