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Short communication

Separation of dihydroxybenzoates, indicators of in-vivo hydroxyl free radical formation, in the presence of transmitter amines and some metabolites in rodent brain, using high-performance liquid chromatography with electrochemical detection

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Abstract

Based on a detailed study of retention parameters, reversed-phase ion-pair chromatographic methods were developed for the simultaneous determination of dihydroxybenzoates, indicators of in-vivo hydroxyl free radical formation, transmitter amines and some metabolites to facilitate neurochemical investigations in rodent brain. Coupling of the separation methods with electrochemical detection and the use of short-chain perfluorinated carboxylic acids for ion-pairing, allowed for a fast and sensitive determination of salicylate-derived 2,3- and 2,5-dihydroxybenzoic acids and the major electroactive, hydroxylated aromatic compounds present in brain samples. Detection limits for the dihydroxybenzoates (signal-to-noise ratio = 2) were 18–22 fmol injected on the column. Basal levels of 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate in the striatum of mice treated with salicylate were 72 ± 13 and 94 ± 11 ng/g wet tissue, respectively.

1. Introduction

Oxygen radicals have been postulated to play a direct or indirect role in several pathological conditions, such as brain ischaemia, Parkinson's disease, arthritis, carcinogenesis, etc. [1,2]. As formation of the highly reactive hydroxyl free radical (HFR) in vivo is difficult to detect, indirect methods have been used to monitor the process. One of the novel methods involves the systematic administration of salicylate, an effective HFR scavenger, which results in the formation of 2,3- and/or 2,5-dihydroxybenzoates (DHBs). Such hydroxylated aromatic compounds can be most sensitively measured using high-performance liquid chromatography with electrochemical detection (HPLC-ED) [3,4].

HPLC-ED methods for the measurement of salicylate-derived DHBs, as main analytes, in human body fluids and gerbil whole brain were described previously [5–7]. In the present work, a detailed reversed-phase ion-pair chromatographic (RP-IPC) retention study was carried

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out to facilitate neurochemical investigations by the simultaneous separation and determination of DHBs, some monoamine transmitters and major metabolites in brain samples. Trifluoroacetate (TFA) or heptafluorobutyrate (HFBA) were used as pairing ion. The effects of pH, the concentrations of the pairing ion, the counter ion and the organic modifier on the separation were studied. Based on the results of the study, this paper describes optimized RP-IPC separation conditions for HFR determination when salicylate is used for the indirect detection of in-vivo free radical generation in brain tissue.

2. Experimental

The HPLC–ED system used for separation and most of the chemicals used were described in an earlier paper [8]. Sodium salicylate, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid and 3,4-dihydroxybenzylamine (DHBA)

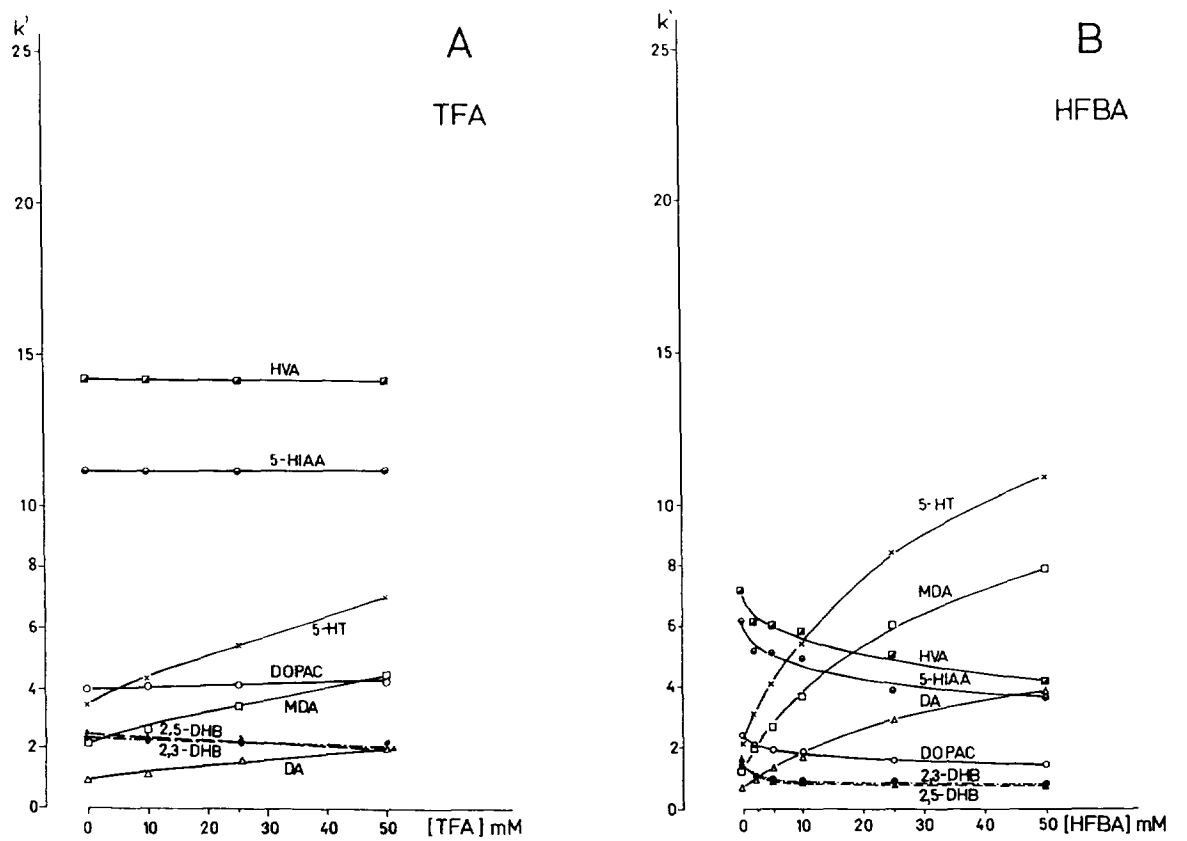

Fig. 1. Dependence of the k' values of some amines, anionic metabolites and dihydroxybenzoates on the concentration of the pairing ion (TFA or HFBA) in the eluent. Column: Nucleosil C_{18} (5 μm), 80 × 4.0 mm I.D.; eluent flow-rate, 0.83 ml/min; detection, amperometric, at 0.75 V. (A) Eluent: aqueous buffer (pH 4.15)–acetonitrile (96.5:3.5, v/v); the aqueous buffer consisted of 125 m*M* Na^+, 0.1 m*M* EDTA and 0–50 m*M* TFA, with the concentration of each component calculated for the whole of the eluent (TFA concentration: 0, 10, 25 or 50 m*M*). Na^+ concentration was set by using a calculated volume of 5 *M* sodium hydroxide solution; the pH of the aqueous buffer was adjusted with 42.5% (w/v) orthophosphoric acid. (B) Eluent: the same as for (A), except that the aqueous buffer to acetonitrile ratio was (93.5:6.5, v/v) and the HFBA concentration was 0, 2, 5, 10, 25 or 50 m*M*.

hydrobromide were purchased from Aldrich (Steinheim, Germany). TFA and HFBA (Sequanal quality) were obtained from Pierce (Rockford, IL, USA). The chromatographic column and the compositions of the mobile phases are specified in the figure captions.

To determine basal levels of 2,3- and 2,5-DHBs in the striata of C57BL mice, tissue samples were prepared in a manner similar to that described in Ref. [8]. Mice were intraperitoneally injected with 100 mg/kg sodium salicylate, and striata were removed 60 min after injection and stored at −80°C until assayed.

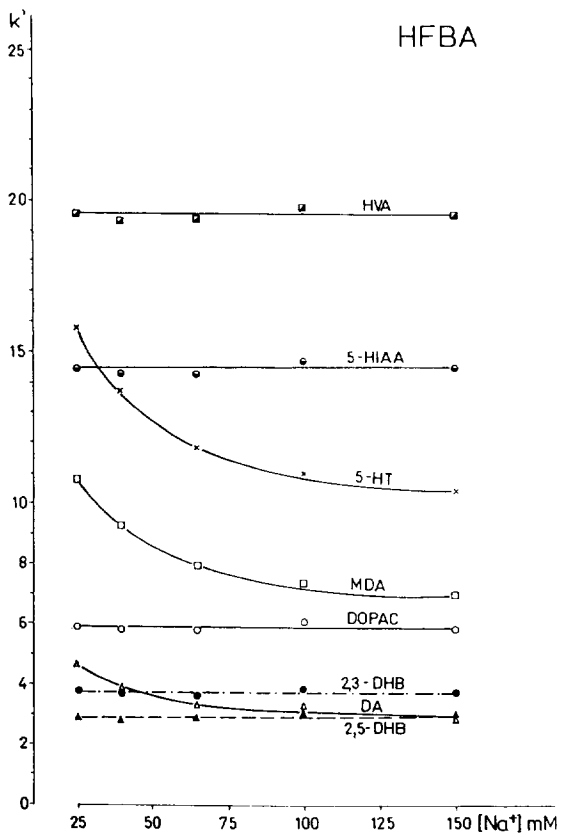

Fig. 2. Dependence of the k' values of some amines, anionic metabolites and dihydroxybenzoates on the concentration of the counter ion (Na^+) in the eluent. Eluent: aqueous buffer (pH 3.50)–acetonitrile (96.5:3.5, v/v); the aqueous buffer consisted of 5 m*M* HFBA, 0.1 m*M* EDTA and 25–150 m*M* Na^+, with the concentration of each component calculated for the whole of the eluent (Na^+ concentration: 25, 40, 65, 100 or 150 m*M*). Other conditions as in Fig. 1.

3. Results and discussion

The dependence of the retention (k' values determined in the usual way) of the compounds examined, i.e. the DHBs, dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenyl-acetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and an internal standard, e.g. α-methyldopamine (MDA) or DHBA, on the major RP-IPC eluent parameters is shown in Figs. 1–3.

Fig. 1 shows the retention as a function of the concentration of the pairing ion in the eluent, TFA (Fig. 1A) or HFBA (Fig. 1B). Fig. 2 demonstrates the influence of the counter ion concentration on the retention in HFBA systems; the dependence of the k' values on the pH of the eluent with TFA or HFBA as pairing ion is shown in Fig. 3A,B. Figs. 1 and 2 show that the retention of amines can be influenced to a higher degree in HFBA systems than in TFA systems (changing the counter-ion concentration in eluents with TFA caused virtually no change in the relative retentions), demonstrating a stronger role of the dynamic ion-exchange in governing the retention in the HFBA systems [9]. As expected, the best way to modify the retention of the acids is by carefully selecting the eluent pH in both systems (Fig. 3). The k' vs. pH plots of the acids in Fig. 3A,B offer good possibilities for the exploitation of the difference in pK_a values of the aromatic and aliphatic carboxylic acids to achieve separation (e.g. the pK_a of 2,3-DHB is 2.94, whereas that of phenylacetic acid is 4.31).

In general, an increase in the concentration of acetonitrile in the eluents (in the 1–7 vol.% range) effectively reduced retention in both systems but gave no changes (TFA systems) or only minor changes (HFBA systems) in the relative retentions.

Fig. 4A shows the separation of the compounds under study in an optimized TFA sys-

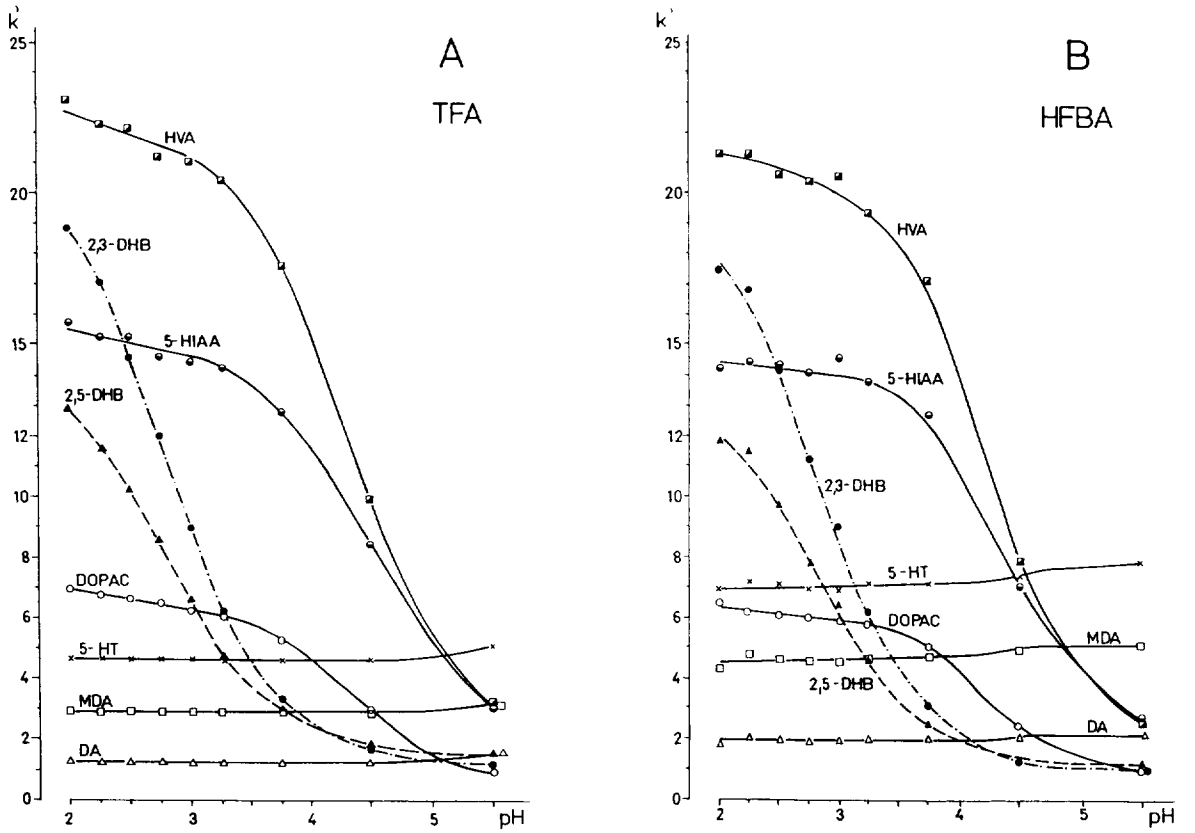

Fig. 3. Dependence of the k' values of some amines, anionic metabolites and dihydroxybenzoates on the pH of the eluent. (A) Eluent: the same as in Fig. 1A, except that TFA concentration was 15 m*M* and the pH of the aqueous buffer was 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.75, 4.50 or 5.50. (B) Eluent: the same as in Fig. 3A, except that HFBA concentration was 3 m*M*. Other conditions as in Fig. 1.

tem, while the separation of the major electroactive compounds in the striatal tissue of mice treated with salicylate is shown in Fig. 4B. Separation of the same samples in an optimized HFBA system is shown in Fig. 5A,B. While both systems allow fast separation and sensitive determination of the DHBs, some differences are worth noting. The earlier elution of the DA metabolites and indole derivatives is an advantage of the TFA system, whereas DA can be better separated from the early eluting sample components in the HFBA system.

The detection limits (signal-to-noise ratio = 2, amount injected) for 2,3-DHB and 2,5-DHB were 3.0–3.5 and 2.5–3.0 pg, respectively, in both systems. The detection limits for the other compounds in the study were specified in Ref. [8]. Basal levels of the DHBs in the striata of mice treated with salicylate under the conditions described in Experimental were 72 ± 13 ng/g wet tissue for 2,3-DHB and 94 ± 11 ng/g wet tissue for 2,5-DHB ($n = 9$). The basal level reported here for 2,5-DHB, assumed to result from in-vivo enzymatic as well as spontaneous hydroxylation of salicylate, is higher than that found under similar conditions in gerbil whole brain by Cao et al. (58.2 ± 8.5 ng/g wet tissue) [7]. The difference might be explained by a higher basal

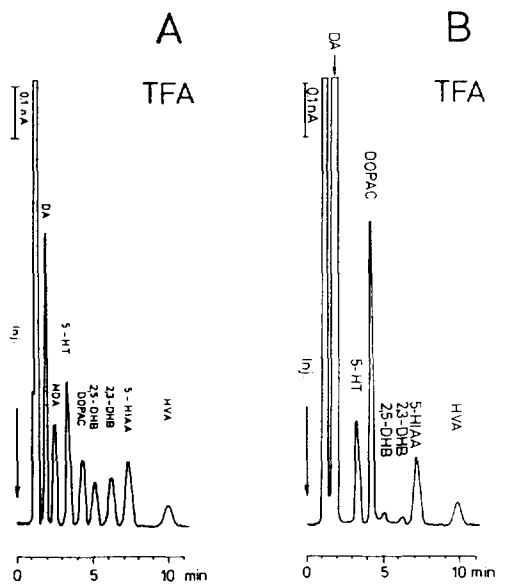

Fig. 4. Separation of some transmitter amines, anionic metabolites and dihydroxybenzoates in an optimized TFA system, using MDA as internal standard. Separations of standards (A), and a centrifuged homogenate prepared with the eluent from the striatal tissue of C57BL mice treated with 100 mg/kg sodium salicylate (i.p.) (B). The eluent was the same as in Fig. 1A, except that TFA concentration was 15 m*M*, Na^+ concentration was 100 m*M* and the pH was 2.75. Other conditions as in Fig. 1.

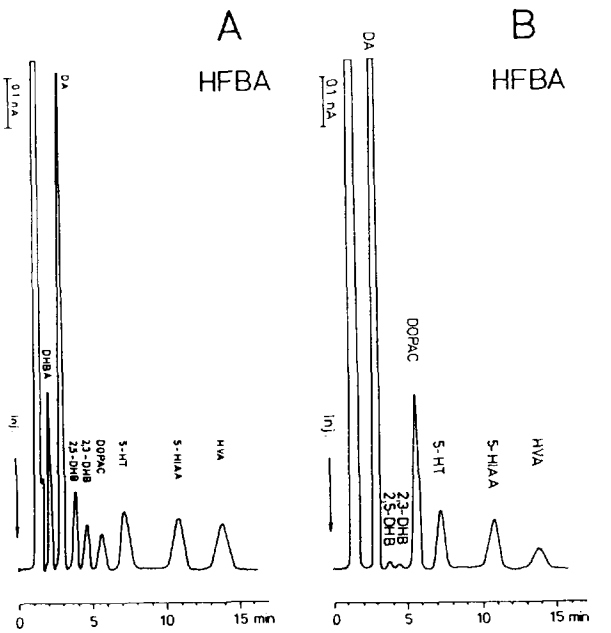

Fig. 5. Separation of some transmitter amines, anionic metabolites and dihydroxybenzoates in an optimized HFBA system, using DHBA as internal standard. Separations of standards (A), and a centrifuged homogenate prepared with the eluent from the striatal tissue of C57BL mice treated with 100 mg/kg sodium salicylate (i.p.) (B). Eluent: aqueous buffer (pH 3.40)–acetonitrile (95:5, v/v); the aqueous buffer consisted of 3 m*M* HFBA, 0.1 m*M* EDTA and 100 m*M* Na^+, with the concentration of each component calculated for the whole of the eluent. Other conditions as in Fig. 1.

level of HFR in striatum associated with the very high DA concentration in this brain region [10]. No basal levels for 2,3-DHB in striatum were reported before.

4. Conclusions

The RP-IPC study of the retention of hydroxylated aromatic compounds presented here using short-chain perfluorinated acids as pairing ions provides a sensitive method for adjusting retention times to achieve the simultaneous separation of salicylate-derived DHBs, indicators of in-vivo HFR formation, transmitter amines and some metabolites in brain tissue. The separation conditions developed as a result of the study allow a faster and more sensitive determination of the DHBs than methods published so far; the detection limits were 18–22 fmol.

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References

[1] I. Fridovich, *Science*, 201 (1978) 875.
[2] B. Halliwell and M.C. Gutteridge, *Biochem. J.*, 219 (1984) 1.

[3] D.M. Radzik, D.A. Roston and P.T. Kissinger, *Anal. Biochem.*, 131 (1983) 458.
[4] R.A. Floyd, J.J. Watson and P.K. Wong, *J. Biochem. Biophys. Methods*, 10 (1984) 221.
[5] M. Grootveld and B. Halliwell, *Biochem. J.*, 237 (1986) 499.
[6] R.-M. Wu, C.C. Chiueh, A. Pert and D.L. Murphy, *Eur. J. Pharmacol.*, 243 (1993) 241.
[7] W. Cao, J.M. Carney, A. Duchon, R.A. Floyd and M. Chevion, *Neurosci. Lett.*, 88 (1988) 233.
[8] M. Patthy and R. Gyenge, *J. Chromatogr.*, 449 (1988) 191.
[9] M. Patthy, *J. Chromatogr. A*, 660 (1994) 17.
[10] C.C. Chiueh, G. Krishna, P. Tulsi, T. Obata, K. Lang, S.-J. Huang and D.L. Murphy, *Free Rad. Biol. Med.*, 13 (1992) 581.