Electrochemical detection of biogenic amines following acylation by N-hydroxysuccinimide esters

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A general methodology for the selective derivatization of amines, to enable quantitation by high pressure liquid chromatography with electrochemical detection, is presented. *N*-Hydroxysuccinimide active esters present in large excess are suitably mild acylating agents to derivatize selectively trace quantities of amines for electrochemical detection. The 2 separate problems of extraction yield and detectability can be solved by this derivatization method. Due to its lipophilicity the resulting *N*-acylated amine, as demonstrated with serotonin, is extracted efficiently into organic solvents during sample preparation for chromatography. Moreover, the acyl group introduced can be designed to be electroactive, thus extending the procedure to amines not readily oxidized, e.g., histamine and phenylethylamine.

Amine *HPLC* *Electrochemical detection* N-*Hydroxysuccinimide ester* *Derivatization* *Bolton-Hunter reagent*

1. INTRODUCTION

Recently we have developed precise, operationally simple procedures [1–3] for the analyses of 3-methoxy-4-hydroxyphenylglycol, homovanillic acid, 5-hydroxyindoleacetic acid, norepinephrine, dopamine and L-dopa in human cerebrospinal fluid (CSF) and plasma using high pressure liquid chromatography (HPLC) with electrochemical detection, and internal standards to achieve accurate quantitation. The speed and reliability of the procedures and the fact that the instrumentation and laboratory manipulations required in these analyses are readily adapted to routine clinical procedures have resulted in growing interest in this methodology. We, therefore, were prompted to extend this approach to devise a derivatization scheme for analyses of a broad spectrum of amines found in very low concentrations in various body fluids. The study of the relationship between concentrations of endogenous amines and their metabolites in CSF and plasma and the functioning of the central nervous system, in particular with regard to mental and neurological disorders [4,5], suggests that a simple, general approach to amine derivatization for electrochemical detection would have multiple applications in neurochemical, endocrine and metabolic fields.

At the outset, we recognized that, in addition to addressing the problems of extraction of trace amounts of a polar amine from aqueous medium and designing suitable internal standards, it would be necessary to develop a strategy which would allow us to detect at low concentrations those amines which are not electroactive, i.e., which would not be amenable to direct electrochemical detection. Joseph and Davies [6] have suggested, but not documented, that a precolumn amine derivatization reaction for amino acids may be used for the detection of amines. A disadvantage of this method is the partial reversibility of the condensation during chromatography. Here we

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demonstrate simple derivatization procedures based on selective acylation by *N*-hydroxysuccinimide esters, which lead to efficient isolation of amine derivatives and which can be used to detect non-electroactive amines using electrochemical detection.

2. MATERIALS AND METHODS

2.1. *Electroactive amines (exemplified by serotonin)*

The derivatization reaction is carried out in 1 ml CSF, pH 11, at 70°C, in the presence of 60 mM acylating reagent (e.g. *N*-succinimidylpropionate). After 2 h the reaction is quenched with excess glycine. The serotonin derivative, **3**, is readily extracted into ethyl acetate and the solvent evaporated. The derivative is injected on an HPLC system as specified in fig.2 and detected using an ESA 5011A electrochemical detector in the oxidative mode with a potential of 0.30 V. The internal standards were prepared by coupling *N-t*-butyloxycarbonyl-5-hydroxytryptophan (Bachem, Torrance, CA) to normal alkyl monoamines (e.g. propyl) using dicyclohexylcarbodiimide/1-hydroxybenzotriazole followed by acidic deprotection, or for the lower homologs by aminolysis of 5-hydroxytryptophan ethyl ester (Sigma, St. Louis, MO).

2.2. *Non-electroactive amines (exemplified by histamine)*

An aqueous solution containing histamine at pH 9.8 is treated with sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate (Pierce, Rockford, IL) at a final concentration of 20 mM, and the solution is shaken vigorously for 30 s at room temperature. The derivative is isolated by ion exchange chromatography (fibrous cellulose phosphate), separated on reversed phase HPLC (Beckman Ultrasphere-ODS column, 25 × 0.46 cm, with a CSK1 guard column packed with Waters Bondapack C_{18}/Corasil; mobile phase – 0.14 M sodium acetate and methanol, 17:73, v/v, containing 3.89 mM 1-octanesulfonic acid and 56 mg/l EDTA, pH 3.48) and detected using an ESA Coulochem model 5100A electrochemical detector set at 0.56 V. Complex interfering peaks are eliminated by a preoxidation at 0.47 V.

3. RESULTS

N-Hydroxysuccinimide esters are used extensively in peptide synthesis and are fairly selective reagents for acylation of amines in the presence of other nucleophiles [7]. We have found that these activated esters readily acylate trace quantities of amines, including neurotransmitters in CSF, in high yield. In cases where the amine contains an electroactive group in the native form, e.g., hydroxyindoleamines and catecholamines, a simple and selective acylation of the amino group results in a less polar derivative, which may be extracted into an organic phase or otherwise separated from ionic species in the biological sample. In cases where the native amine is not sufficiently electroactive, a similar acylation using an *N*-hydroxysuccinimide ester of an electroactive carboxylic acid leads to a derivative which may be detected electrochemically. This dual strategy of acylation of amines selectively for electrochemical detection is outlined in fig.1. Examples of both types of analyses are presented.

Despite many reported methods for the detection of serotonin [8–10] its quantitation in CSF has been seriously hampered due to difficulties in extracting this amine from aqueous media [11]. We have measured serotonin, **1a**, with a reproducible detection limit of 0.1 pmol/ml CSF (18 parts per trillion) following selective derivatization with *N*-succinimidyl propionate (**2**, R_2 = H, A = Et) as shown in fig.2 [12]. Any *N,O*-diacylated species formed (diacylation occurs to a much lesser degree under these conditions compared to acylation with propionic anhydride) are hydrolyzed to the monoacyl derivative, **3a**, during the heating. The *N*-propionyl derivative was selected because the lower homolog, *N*-acetylserotonin, was found to be present in considerable quantity in samples of human and non-human primate CSF [13]. A typical HPLC trace derived from a CSF sample found to contain 1.4 pmol serotonin/ml is shown (fig.2).

The implicit variability between experiments necessitates the use of an internal standard having polarity and reactivity very similar to serotonin. A series of *N*-alkyl amides, **1b**, of 5-hydroxytryptophan readily fulfilled this purpose. The number of methylene units (*m*) may be varied to alter the reverse phase HPLC retention time so

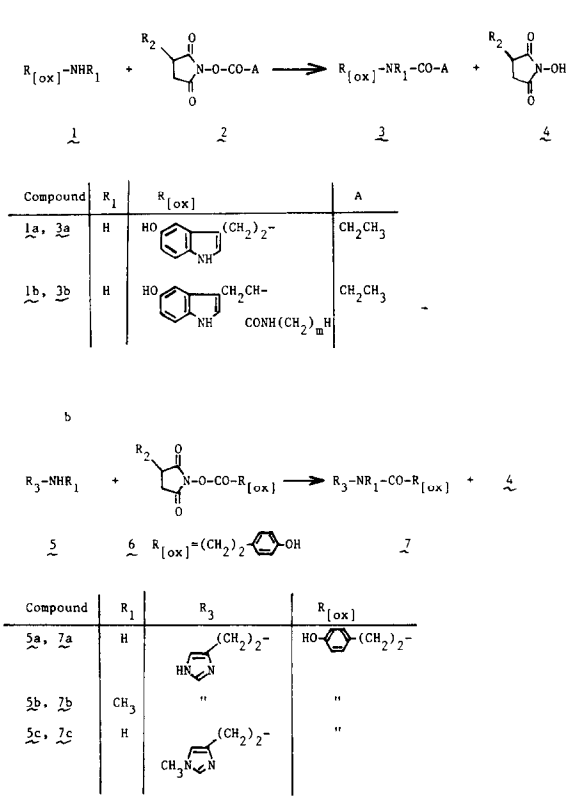

Fig.1. Selective derivatization of amines for electrochemical detection ($R_{[ox]}$ is an oxidizable group, such as a phenol; R_2 is H or SO_3Na; A is a non-electroactive group, e.g. Me, Et). (a) Amines bearing oxidizable groups. (b) Non-electroactive amines.

that interfering peaks present in the sample may be avoided. For studies of monkey and human CSF, the optimum length is $m = 3$, i.e. the *N*-propyl amide of 5-hydroxytryptophan. Using standardized samples ($n = 12$), we have determined that derivatization and extraction of both serotonin and the internal standard occur with an overall recovery of 53 ± 3% (65 ± 5% for extraction alone, $n = 18$). It can be seen that the internal standard is less polar than the serotonin derivative and that it is well separated from other peaks (fig.2).

It should be noted that all primary and secondary amines present in the CSF are susceptible to acylation during the derivatization step. Thus, a peak (a) co-chromatographing with the *N*-propionyl derivative of normetanephrine, a major metabolite of norepinephrine, was observed. To

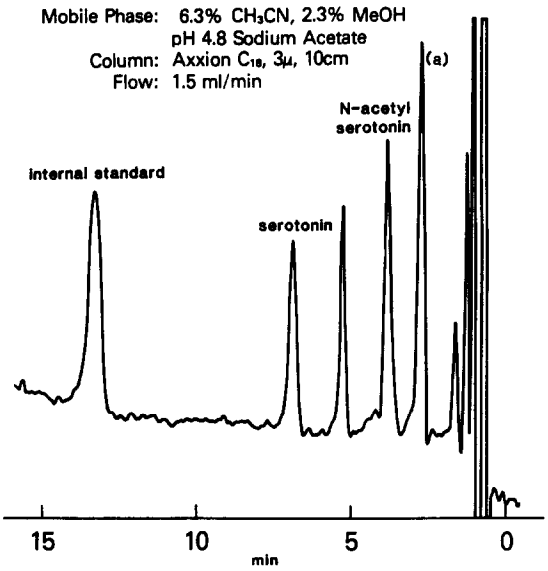

Fig.2. Chromatogram obtained after treatment of human cerebrospinal fluid with *N*-succinimidyl propionate and extraction into ethyl acetate.

explore the possibility of extending the method to other electroactive amines a set of biogenic amine standards was derivatized using *N*-succinimidyl propionate (table 1). The expected monoacyl products were isolated by ethyl acetate extraction and identified by mass spectroscopy. The derivatives were separable and detectable electrochemically under similar chromatographic conditions. The tyramine derivative was observed when the reaction was carried out on an 'artificial CSF' containing human serum albumin. Thus, the selective acylation approach using *N*-hydroxysuccinimide active esters is potentially applicable to the trace determination of many other primary and secondary phenolic amines.

The analysis of histamine, **5a**, relies on the dual strategy described above, i.e. the attachment of an electroactive group through mild and selective acylation. Biological samples containing histamine were derivatized [14] using the *N*-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)propionic acid, **6**, R_2 = H. This compound, also known as the Bolton-Hunter [15] or Rudinger reagent [16], is used widely as a prosthetic group which can be attached to proteins for radioiodination. The acylation occurs even more efficiently using the commercially available water soluble 'sulfo' analogue

Table 1

Selective *N*-acylation of various phenols by *N*-succinimidyl propionate

Parent amine	Ion observed in mass spectrum[a]	Retention time[b] (min)
Dopamine	210[c]	9.2
Epinephrine	222[d]	9.0
Metanephrine	236[d]	19.3
3-Methoxytyramine	214[c]	23.4
Norepinephrine	208[d]	3.3
Normetanephrine	222[d]	6.0
Octopamine	210[c]	4.6
Serotonin	233[c]	17.6
Tyramine	194[c]	17.0

[a] Chemical ionization with ammonia gas

[b] Mobile phase: sodium acetate buffer (0.1 M, pH 4.8) containing 6.3% acetonitrile, 2.3% methanol, and 0.01% EDTA; column: Supelco RP, LC-8, 3 micron particle size, 4.6 mm × 15 cm; flow 1.3 ml/min, 3500 lb/inch^2

[c] Equals $M_r + 1$

[d] Equals $M_r - 18 + 1$, due to elimination of water

of **6** in which $R_2 = SO_3Na$ [17]. Pure samples of the histamine derivatives, **7a–c**, were prepared for comparison. N^{α}-Methylhistamine, **5b**, was used as an internal standard after it was shown rigorously that we could detect no endogenous N^{α}-methylhistamine [18] in rat hypothalamus tissue. Reactions of histamine, N^{α}-methylhistamine and N^{τ}-methylhistamine (**5c**), which was detected endogenously, were carried out with 90% efficiency. The 3 derivatives were resolved by HPLC and the peak height was linear with the original amine concentration. Using this procedure concentrations of histamine in single nuclei of the rat hypothalamus were determined. It was possible to measure the level of histamine in as little as 1 mg brain tissue.

Compound **6** was used to derivatize other non-electroactive amines such as tryptamine, and the derivatives were separable by HPLC and detectable by electrochemical methods.

4. DISCUSSION

The biological importance of many amines has spurred intense interest in the development of precise and sensitive methods [9–11,18,19] for quantitation of these substances. This quantitation presents unique challenges because of the very low concentrations often encountered and because of the difficulties in isolating and concentrating these usually polar compounds in a reproducible and efficient manner from the aqueous milieu. The use of mass spectroscopy by Markey and coworkers [11] coupled with the preparation of deuterated standards has proved to be a very useful technique. Unfortunately, the involved sample preparation, the high cost of instrumentation and very special personnel requirements preclude the use of this approach in most research programs. As we have shown here, HPLC in combination with precolumn derivatization methods, enabling efficient recovery and highly sensitive electrochemical detection, represents an alternative approach to this problem. These findings suggest the generality of the parallel approaches for derivatization of electroactive and non-electroactive amines to a wide variety of amines, which in certain combinations can be determined simultaneously.

REFERENCES

[1] Scheinin, M., Chang, W.-H., Kirk, K.L. and Linnoila, M. (1983) Anal. Biochem. 131, 246–253.

[2] Scheinin, H., Chang, W.-H., Jimerson, D.C. and Linnoila, M. (1983) Anal. Biochem. 132, 165–170.

[3] Brown, R.T., Oliver, J., Kirk, K.L. and Kopin, I.J. (1984) Life Sci. 34, 2313–2318.

[4] Asberg, M., Thoren, P., Traskman, L., Bertilsson, L. and Ringberger, V. (1976) Science 191, 478–480.

[5] Linnoila, M., Virkkunen, M., Scheinin, M., Nuutila, A., Rimon, R. and Goodwin, F.K. (1983) Life Sci. 33, 2609–2614.

[6] Joseph, M.H. and Davies, P. (1983) J. Chromatogr. 277, 125–136.

[7] Anderson, G.W., Zimmerman, J.E. and Callahan, F.M. (1963) J. Am. Chem. Soc. 85, 3039.

[8] Narasimhachari, N. (1984) J. Liq. Chromatogr. 7, 2679–2689.

[9] Anderson, G.M. and Young, J.G. (1981) Life Sci. 28, 507–517.

[10] Turner, W.J. and Mauss, E.A. (1959) Arch. Gen. Psychiatry 1, 108–112.

[11] Markey, S.P., Colburn, R.W. and Johannesen, J.N. (1981) Biomed. Mass Spectrosc. 8, 301–304.

[12] Linnoila, M., Jacobson, K.A., Marshall, T., Miller, T.L. and Kirk, K.L. (1985) Life Sci., submitted.

[13] Taylor, P.A., Garrick, N.A., Tamarkin, L., Murphy, D.L. and Markey, S.P. (1985) Science 228, 900.
[14] Mine, K., Jacobson, K.A., Kirk, K.L., Kitajima, Y. and Linnoila, M. (1985) Anal. Biochem., submitted.
[15] Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–538.
[16] Rudinger, J. and Ruegg, U. (1973) Biochem. J. 133, 538–539.
[17] Staros, J.V. (1982) Biochemistry 21, 3950–3955.
[18] Wollin, A. and Navert, H. (1985) Anal. Biochem. 145, 73–79.
[19] Udenfriend, S., Stein, S., Boehlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Science 178, 871–872.