Journal of Chromatography, 489 (1989) 371–376
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4627

Note

Electrochemical label for high-performance liquid chromatography

I. β-Naphthoquinone-4-sulphonate as an electrochemical detection labelling reagent of amines

YUJI NAKAHARA*, AKIKO ISHIGAMI and YASUSHI TAKEDA

National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo (Japan)

(First received August 1st, 1988; revised manuscript received November 2nd, 1988)

Electrochemical detection (ED) with high-performance liquid chromatography (HPLC) offers considerable improvements in sensitivity and selectivity over the UV absorption method. Its usefulness has been demonstrated in the analysis of various classes of compounds, mostly catechols, aromatic amines and phenols, but it is limited to electrochemically active compounds.

Recently, several derivatization reagents of potential utility for HPLC–ED of amines have been reported, such as trinitrobenzenesulphonic acid [1], N-hydroxysuccinimide of homovanillic acid [2], N-4(4-anilinophenyl)isomaleimide and N-(4-anilinophenyl)isophthalimide [3], *p*-N,N-dimethylaminophenyl isothiocyanate [4] and *o*-phthalaldehyde [5]. However, their use made lead to some practical problems, such as high background, side-reactions, lack of stability, reactivity, non-availability and the need for complex pretreatment.

Sodium β-naphthoquinone-4-sulphonate (BNQS) has been used as a colouring reagent for amines in thin-layer chromatography [6]. β-Naphthoquinone (BNQ) derivatives would be suitable for sensitive electrochemical reduction, and the BNQ-labelling reaction can be carried out in aqueous solution. The BNQ derivatization is a substitution reaction for primary and secondary amines, as shown in Fig. 1. The BNQ derivatives would be electrochemically reduced under acidic conditions to give 1,2-dihydroxynaphthylamine derivatives with an accompanying uptake of two electrons (Fig. 1).

This paper describes the application of BNQS to labelling of amines for HPLC–ED.

0378-4347/89/$03.50

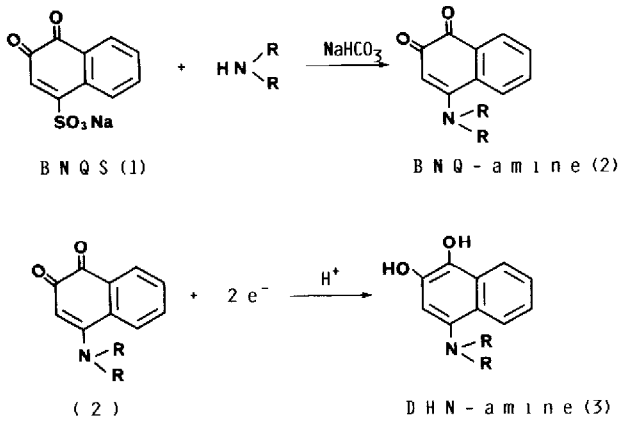

Fig. 1. Reaction of β-naphthoquinone sulphate (1) with amines and the electrochemical reduction of 4-amino-β-naphthoquinone.

EXPERIMENTAL

Reagents

BNQS was purchased from Nakarai Chemicals (Kyoto, Japan) and recrystallized five times from 80% aqueous ethanol. A solution of 0.5% BNQS in distilled water was prepared just before use. Methamphetamine (MA) hydrochloride and ephedrine hydrochloride were purchased from Dainippon Pharmaceutical (Osaka, Japan). β-Phenethylamine (PEA) hydrochloride, phenylpropanolamine hydrochloride, mescaline (Ms) sulphate, benzylethylamine hydrochloride, N-methylphenethylamine hydrochloride and dibenzylethylenediamine hydrochloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The other phenethylamines were synthesized by the methods reported in the literature (e.g. refs. 7 and 8).

All solvents were distilled and filtered through Millex-FG50 (Millipore, Bedford, MA, U.S.A.) before use.

Instruments and chromatographic conditions

Chromatography was carried out with an Irica Σ-871 liquid chromatograph (Irica Kogyo, Kyoto, Japan), a Rheodyne injector, a μBondasphere C_{18} column (5 μm, 150 mm$\times$3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.), an Irica Σ-875 amperometric detector with a glassy carbon electrode (Irica Kogyo) and a Shimazu Chromatopack C-R3A data recorder (Shimazu, Kyoto, Japan). The working electrode was set at a potential of -0.0 V (vs. Ag/AgCl). Elution was carried out with acetonitrile–methanol–0.01 *M* sulphuric acid (20:20:70, v/v) at a flow-rate of 1.1 ml/min. All separations were performed at 40°C.

Standard procedure for BNQ derivatization of amines

To a solution of 100 μl of aqueous sample containing the amine (<100 μg/ml) were added 100 μl of internal standard (I.S.) solution [methoxyphenamine (MOP), 100 μg/ml], 200 μl of 2% sodium bicarbonate and 200 μl of 0.5% BNQS.

Then the reaction mixture was heated in a capped glass vial at 60°C for 40 min. After cooling, the reaction mixture was extracted with 1 ml of hexane–ethyl acetate (1:1, v/v) by vortex-mixing for 1 min. The organic layer was transferred into a 10-ml glass tube with a Pasteur pipette and evaporated to dryness under nitrogen stream. The residue was dissolved in a 100 μl of methanol and a 5-μl sample was injected into the chromatograph.

Preparation of BNQ derivatives

To 2 ml of phenethylamine hydrochloride or sulphate (10 mg/ml in water), 10 ml of 1% sodium bicarbonate and 40 ml of 0.5% BNQS were added, and the mixture was heated at 70°C for 1 h. The reaction mixture was extracted with chloroform and evaporated to dryness. The crude products were purified by column chromatography on silica gel using benzene–acetone–ethanol (70:30:5, v/v) and then recrystallized from ethanol. The purity of the BNQ derivatives obtained was confirmed by comparison with the melting points and the molar absorptivities (ϵ) reported by Hashimoto et al. [6].

RESULTS AND DISCUSSION

Optimization of labelling

Variables such as reagent ratio, concentration of sodium bicarbonate, temperature and reaction time were optimized for the labelling of amines in aqueous solution. The optimum molar ratio of labelling reagent to base to amine was 100:8:1. No improvement in peak height was obtained when the relative proportion of labelling reagent was greater than 100. Since the unreacted reagent is not extracted with hexane–ethyl acetate (1:1), a large molar excess of reagent caused little interference. When the ratio of the labelling reagent to the base was less than 10:1, the undesired peaks resulting from the reagent increased. When the ratio of base to amine was less than 8:1, the chromatogram showed that peak response decreased somewhat.

Other factors that influence the degree of BNQ labelling are the temperature and the reaction time. The BNQ labelling of MA was carried out at 40, 50, 60 and 80°C at which temperatures it took 90, 60, 40 and 40 min to reach the plateau, respectively. The time course of BNQ labelling of MA, amphetamine (AP), MOP, phentermine (PTM) and *p*-hydroxymethamphetamine (OHMA) was studied at 60°C until the derivatization reached the plateau. All BNQ labelling reached the plateau by 40 min.

Removing excess reagent and undesired compounds

When the reaction mixture was injected directly onto the column, the chromatogram showed many peaks that interfered with the peaks of the BNQ derivatives. Therefore, an extraction procedure was needed to remove excess reagent and undesired compounds. The effect of the extraction solvent on this procedure was examined. Hexane, diethyl ether, benzene, ethyl acetate, hexane–ether (1:1, v/v), chloroform and hexane–ethyl acetate (1:1, v/v) were all tried, and the best solvent for the recovery of BNQ derivatives from biological specimens was found to be chloroform. Its use afforded good recoveries of polar compounds; however,

more compounds from body fluids were extracted. Although the recoveries of BNQ-EP, BNQ-Ms, BNQ-OHAP and BNQ-OHMA with hexane– ethyl acetate (1:1, v/v) were 72–85%, the other BNQ derivatives were quantitatively extracted with hexane–ethyl acetate (1:1, v/v). Therefore, we selected hexane–ethyl acetate (1:1, v/v) as the extracting solvent for the standard method.

Optimum working voltage

For determining the optimum working voltage, a hydrodynamic voltammogram of BNQ-MA was studied from 0.3 to −0.1 V. Fig. 2 shows that the highest response for BNQ-MA was obtained at ca. 0.0 V.

Standard curve and detection limit

The standard curves for BNQ derivatives were linear between 10 and 10 000 ng/ml. The linear regression lines were $y=0.482x+0.001$ ($r=0.995$) for BNQ-MA and $y=0.572x+0.003$ ($r=0.996$) for BNQ-AP (x=concentration of drugs in μg/ml of aqueous solution, y=peak-area ratio of BNQ derivatives to BNQ-I.S.). The within-day coefficients of variation (C.V.) ($n=10$) of this analysis for phenethylamines in aqueous solution were 2.5–3.6% at 100–10 000 ng/ml.

In order to determine the detection limits, a series of standard solutions containing BNQ-Ms, BNQ-PEA and BNQ-AP was prepared. The injected solutions contained the equivalent of 0–100 pg (0–0.3 pmol) per 10-μl injection. The detection limit was 10 pg (0.03 pmol) based on a signal-to-noise ratio of 3. Fig. 3 shows the chromatograms of BNQ-Ms, BNQ-PEA and BNQ-AP from 10 to 100 pg per injection.

The retention times of the BNQ derivatives of 21 phenethylamines are given in Table I: a good separation was obtained within 38 min.

The BNQ derivatives of AP, MA, MOP, PTM and 2,5-dimethoxy-4-methylamphetamine (STP) were clearly separated from each other and the endogenous compounds, as shown in Fig. 4A. Fig. 4B shows the chromatogram obtained by BNQ labelling using a urine sample containing OHMA, AP, MA and PTM.

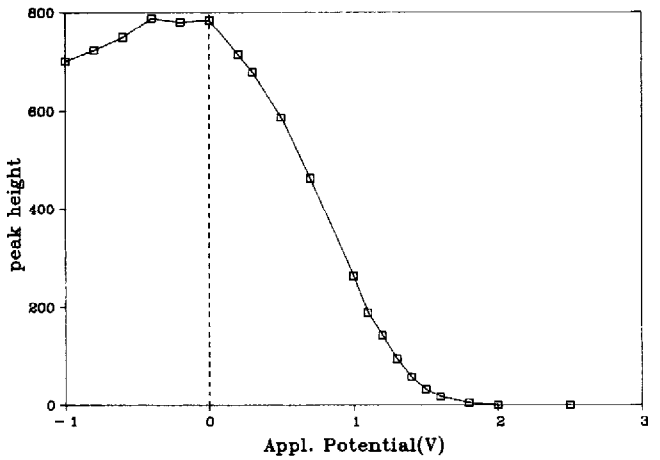

Fig. 2. Hydrodynamic voltammogram, with 20 ng of BNQ-MA injected.

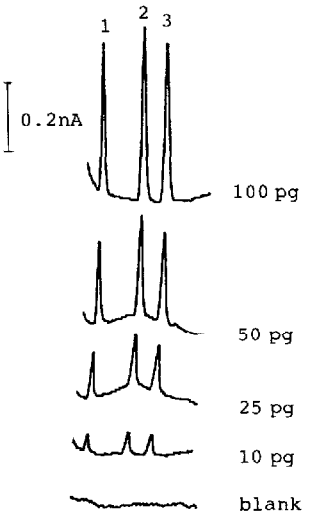

Fig. 3. Chromatograms of injections of 10–100 pg of BNQ-Ms, BNQ-PEA and BNQ-AP under the conditions described in the text. Peaks: 1 = BNQ-Ms; 2 = BNQ-PEA; 3 = BNQ-AP

TABLE I

RETENTION TIMES OF BNQ DERIVATIVES

Compound	Retention time (min)	Relative retention time (MA = 1)
Ephedrine	4.22	0.26
p-Hydroxyamphetamine	4.56	0.29
Phenylpropanolamine	5.55	0.35
Mescaline	6.24	0.39
3,4,5-Trimethoxyamphetamine	6.65	0.42
p-Hydroxymethamphetamine	7.07	0.44
β-Phenethylamine	10.26	0.64
3,4-Methylenedioxyamphetamine	11.00	0.69
N-Methyl-*β*-phenethylamine	11.66	0.73
Chlorephedrine	11.82	0.74
m-Methoxyamphetamine	12.36	0.77
Amphetamine	12.86	0.81
p-Methoxyamphetamine	15.50	0.97
Methamphetamine	15.97	1.00
o-Methoxyamphetamine	19.05	1.20
Methoxyphenamine	20.27	1.26
N-Benzylethylamine	21.95	1.37
Dibenzylethylenediamine	23.22	1.45
Phentermine	23.91	1.50
2,5-Dimethoxy-4-methylamphetamine	34 48	2.16
4-Bromo-2,5-dimethoxyamphetamine	37.14	2.33

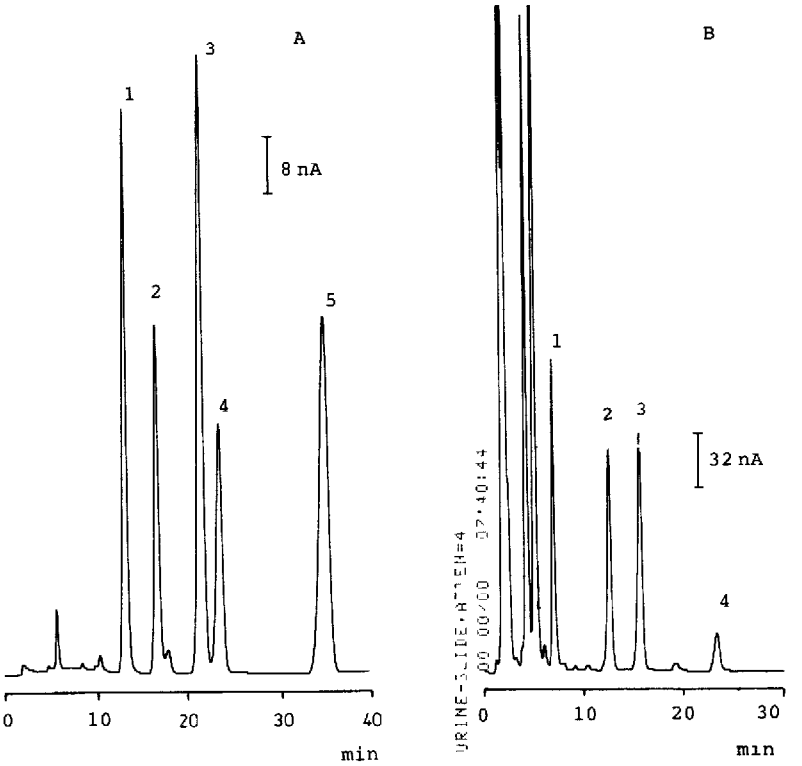

Fig. 4. (A) Chromatogram of BNQ labelling of a solution containing 100 ng/ml AP, MA, MOP, PTM, and STP. Peaks: 1=BNQ-amphetamine; 2=BNQ-methamphetamine; 3=BNQ-methoxyphenamine; 4=BNQ-phentermine; 5=BNQ-STP. (B) Chromatogram of BNQ labelling of urine containning OHMA, AP, MA and PTM at 1 μg/ml. Peaks: 1=BNQ-OHMA; 2=BNQ-amphetamine; 3=BNQ-methamphetamine; 4=BNQ-phentermine.

In conclusion, the use of BNQS as an ED label for amines satisfies the requirements of a good labelling reagent, in terms of sensitivity, selectivity, short reaction time, reproducibility, simple sample pretreatment and low background. The use of 0 V as an applied potential leads to a stable and clean cell and makes the background current lower. Our method makes possible the direct analysis of urine and plasma without the tedious extraction procedures.

REFERENCES

1 W.L. Caudill and R.M. Wightman, Anal. Chim. Acta., 141 (1982) 269.
2 K. Shimada, M. Tanaka and T. Nambara, Chem. Pharm. Bull., 27 (1979) 2259.
3 K. Shimada, M. Tanaka and T. Nambara, J. Chromatogr., 280 (1983) 271.
4 T.J. Mahachi, R.M. Carlson and D.P. Poe, J. Chromatogr., 298 (1984) 279.
5 L.A. Allison, G.S. Mayer and R.E. Shoup, Anal. Chem., 56 (1984) 1089.
6 Y. Hashimoto, M. Endo, K. Tominaga, S. Inuzuka and M. Moriyasu, Mikrochim. Acta, (1978) 493.
7 A. Buzas and C. Dufor, Bull. Soc. Chim. France, (1950) 139.
8 M. Ohno, M. Shimamine and K. Takahashi, Bull. Nat. Inst. Hyg. Sci., 91 (1973) 41.