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Note

High-performance liquid chromatography with electrochemical detection for the determination of nicotine and N-methylnicotinium ion

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The determination of nicotine and its metabolites is of particular interest in studying the correlation between the biological effects of tobacco smoking and the pharmacodynamics and pharmacokinetics of nicotine, the principal pharmacologically active component of tobacco.

Previous studies have shown that nicotine is primarily metabolized in humans to cotinine and nicotine-N'-oxide[1], both metabolites being formed via independent oxidation pathways[2]. However, nicotine methylation has also been reported[3–6], and recent studies indicate that N-methylnicotinium ion, the major methylated urinary metabolite of nicotine, is formed from an S-adenosylmethionine-dependent methyltransferase enzyme system that is widely distributed in tissues[7–9]. Also N-methylnicotinium ion has the potential to function as an *in vivo* methylating agent[10], and there is evidence that this metabolite is subsequently biotransformed *in vivo* in both mouse and rat, to nicotine[11]. Thus, we were interested in the development of a sensitive and selective assay for the determination of both nicotine and N-methylnicotinium ion.

Several analytical techniques for the quantitation of nicotine and its oxidation metabolites in biological fluids have been described in the literature. However, these methods generally lack the required sensitivity and selectivity for measuring the low levels of these substances in the urine and plasma of smokers, and no current method is available for the determination of the low levels of polar, water-soluble N-methylated quaternary metabolites of nicotine. The most commonly used techniques employed involve solvent extraction of nicotine followed by gas chromatography–mass spectrometric analysis[12–19], or liquid chromatography with ultraviolet spectrophotometric detection[20,21]. However, these procedures are not suitable for the determination of the non-volatile, non-organic soluble, N-methylated quaternary nicotine metabolites.

The use of liquid chromatography coupled with electrochemical detection (ED) has been widely and successfully applied to the detection of oxidizable or reducible

compounds such as catecholamines and other amines[22]. We have utilized the chemistry of nicotine and its metabolites for the selective analysis of nicotine and its N-methylated metabolite by coulometric ED. We now report an analytical method for the determination of both nicotine and N-methylnicotinium ion by high-performance liquid chromatography (HPLC) and coulometric detection with sensitivity in the picogram range.

MATERIALS AND METHODS

Reagents and standards

S(−)-Nicotine (Fig. 1, structure 1) was purchased from Aldrich (St. Louis, MO, U.S.A.) and was distilled under vacuum before use; the N-methylnicotinium iodides (Fig. 1, structures 2–4) (X = I) were prepared by the procedure of Seeman and Whidby[23].

HPLC-grade acetonitrile, methanol, 85% phosphoric acid and sodium dihydrogen phosphate were obtained from Fisher Scientific (Pittsburg, PA, U.S.A.); sodium octyl sulfate was obtained from Kodak (Rochester, NY, U.S.A.). All buffers were prepared in preboiled, double distilled water. The solutions were then cooled and filtered under vacuum through a 0.22-μm filter (type GS) followed by degassing using sonication under vacuum.

Equipment

Analyses were carried out on a HPLC system (Waters Assoc., Milford, MA, U.S.A.) comprising a Model 6000A solvent delivery system, a Model U6K Universal injector unit and data module recorder and integrator, Model 730. Chromatographic separations were carried out on a reversed-phase μBondapak C_{18} column (30 × 0.39 cm I.D., 5 μm particle size) (Waters Assoc.). Elution of solutes was monitored with a Coulometric Electrochemical Detector, Model 5100A fitted with a dual electrode assembly (ESA, Bedford, MA, U.S.A.).

Chromatographic conditions

Separations were effected using an isocratic mobile phase consisting of a pri-
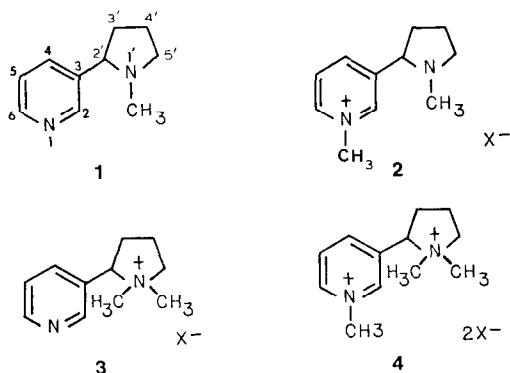

Fig. 1. Structural formulae for nicotine and N-methylated derivatives: 1 = nicotine; 2 = N-methylnicotinium ion; 3 = N'-methylnicotinium ion; 4 = N,N'-dimethylnicotinium ion.

mary mobile phase of 2 mM sodium dihydrogen phosphate containing 0.25 mM sodium octyl sulfate (92.5–95.0%) and a secondary mobile phase of acetonitrile–methanol (3:1) (7.5–5.0%), adjusted to pH 3.0 with phosphoric acid; for appropriate conditions see legend to Fig. 2. Analyses were performed at room temperature and the analytical column was equilibrated with mobile phase before connecting to the detector. After connecting the detector, the system was equilibrated overnight before applying samples.

Optimization of chromatographic performance

The optimum parameters selected for the use of the coulometric ED system were: an applied reduction potential of −0.50 V and an oxidation potential of +0.75 V. A pulse dampener, a good grounding, a low molarity of electrolyte, and the application of an oxidation potential in excess of +1.00 V through the guard cell for oxidation of the mobile phase and reduction of background noise, afforded improved performance and enhanced detector sensitivity and stability.

RESULTS AND DISCUSSION

Chromatographic separation of nicotine and N-methylnicotinium ion from the oxidation metabolites cotinine, 3-hydroxycotinine and nicotine-N'-oxide, was easily achieved (see Table I and Fig. 2) using mixtures of 90–95% primary buffer, and 5–10% secondary buffer in the isocratic mode. A convenient system was developed using 92.5% primary mobile phase and 7.5% secondary mobile phase at a flow-rate of 1.20 ml/min, isocratically (see Table I and Fig. 2).

In the analysis of nicotine and N-methylnicotinium ion by ED, it is likely that the electrochemical reaction involves a one electron transfer with an irreversible secondary reaction involving solvent, as has been observed for other tertiary amino compounds[24–26]. It is postulated that nicotine initially loses a lone pair electron from the pyrrolidine ring N to afford a cation radical, which then rapidly extracts a proton from the solvent (see Fig. 3). The presence of acetonitrile as a solvent component appears to be important, as has been previously reported[27], since it is likely that it

TABLE I

ELECTROCHEMICAL DETECTABILITY AND RETENTION TIMES OF NICOTINE AND RE-LATED COMPOUNDS

Compound	Electrochemical detectability	Retention time (min) (UV 254 nm)
Nicotine	Yes (0.1 ng)*	16.1
Cotinine	No	4.5
Nicotine N'-oxide	No	2.5
3-Hydroxycotinine	No	2.5
N-Methylnicotinium ion	Yes (0.2 ng)*	18.0
N'-Methylnicotinium ion	No	—
N,N'-Dimethylnicotinium ion	No	—

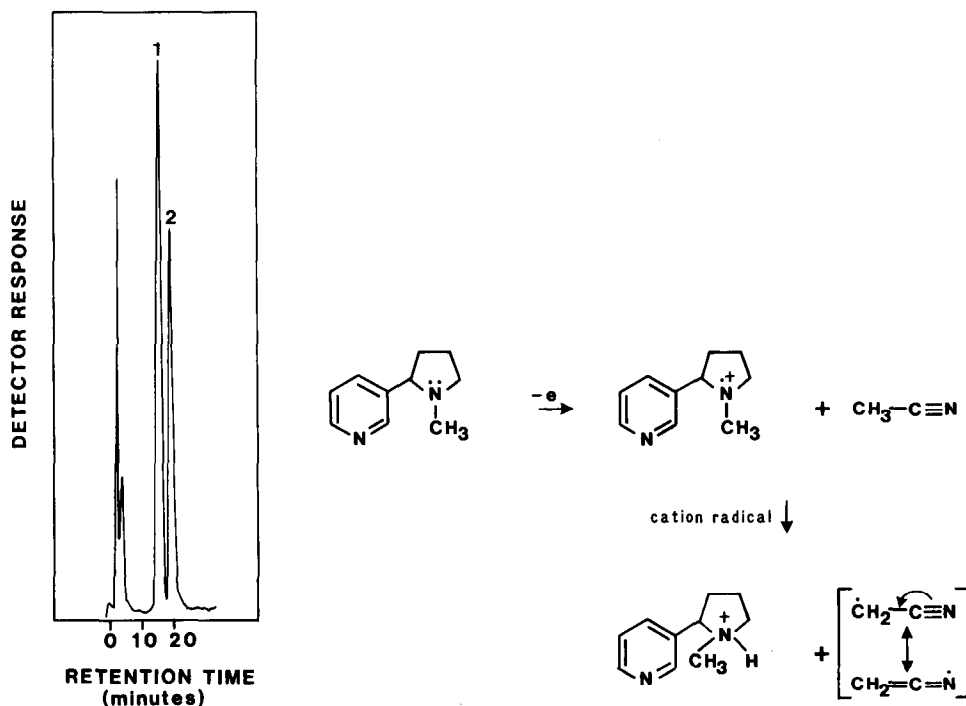* Limits of detection at a signal-to-noise ratio of 4:1.

Fig. 2. Separation of nicotine (1) and N-methylnicotinium ion (2) by reversed-phase HPLC with ED. Conditions: primary buffer, 92.5% 2 mM NaHPO$_4$ containing 0.25 mM sodium octyl sulfate; secondary buffer, 7.5% methanol–acetonitrile (3:1) adjusted to pH 3.0 with phosphoric acid; flow-rate 1.20 ml per min; column, reversed-phase μBondapak C$_{18}$, 5 μm particle HPLC (30 × 0.39 cm I.D.). See Materials and methods section for electrochemical detector parameters.
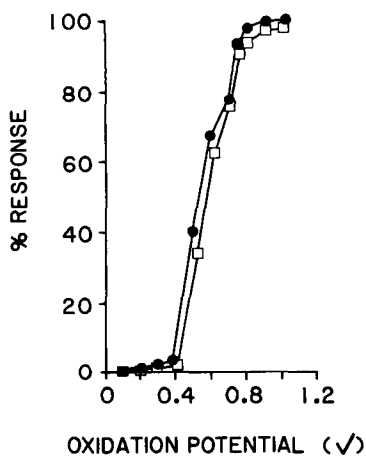
Fig. 3. Electrochemical oxidation of nicotine.

Fig. 4. Relationship between applied oxidation potential and detector response for nicotine (●) and N-methylnicotinium ion (□).
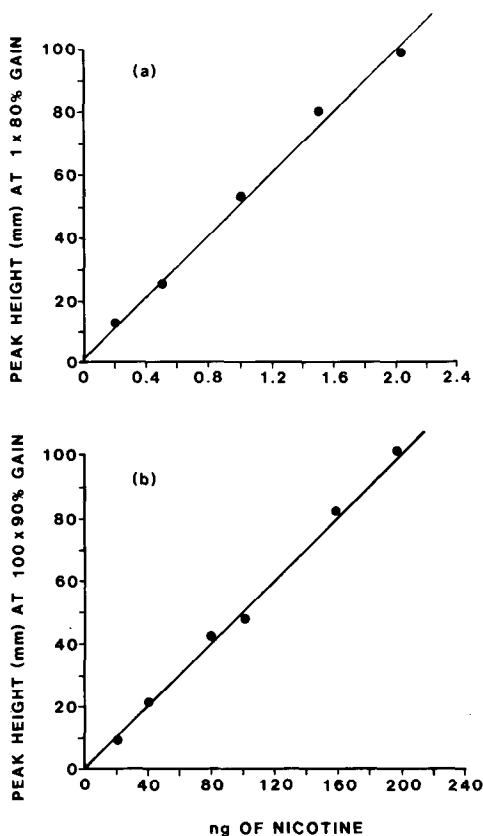
Fig. 5. Nicotine standard curves in the range (a) 0.2–2.0 ng, and (b) 20–200 ng. Values are means of at least three determinations; correlations of 0.9965 and 0.9987 were obtained for curves (a) and (b) respectively.

is the active species in the solvent reaction, generating a resonance stabilized radical. The resulting protonated amine is not reducible and is electroinactive. The observation that N-methylnicotinium ion (Fig. 1, structure 2) affords a level of detection almost comparable with nicotine, whereas N'-methylnicotinium ion (Fig. 1, structure 3) and N,N'-dimethylnicotinium ion (Fig. 1, structure 4) are both insensitive to ED using the conditions described above (see Table I), is strong evidence to support the involvement of the pyrrolidine N, and not the pyridine N lone pair of electrons in the oxidation mechanism.

Fig. 4 illustrates the relationship between the oxidation potential applied and the percentage response obtained after injection of a constant amount of nicotine and N-methylnicotinium iodide. For optimum conditions, an oxidation potential of +0.75 V was found to be most suitable.

Calibration curves for nicotine and N-methylnicotinium ion showed linearity over the concentration range 0.2 ng to 5 μg. Nicotine standard curves are illustrated in Fig. 5a and b, over the range 200 pg to 200 ng. Good reproducibility was obtained for repeat injections; similar sensitivity and wide range linearity were observed with N-methylnicotinium ion (data not shown). The oxidation metabolites, cotinine, 3-

hydroxycotinine and nicotine N'-oxide were not observed in the above sensitivity range by ED using the conditions employed for the nicotine analysis.

CONCLUSION

A sensitive and selective HPLC system with ED, has been developed for the analysis of nicotine and N-methylnicotinium ion down to the upper picogram level. This analytical procedure should be of utility in quantitating the levels of the above compounds in the blood, tissue and urine of animals and humans that have been exposed to nicotine in tobacco smoke.

ACKNOWLEDGEMENTS

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