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COMPARISON OF ELECTROCHEMICAL DETECTION METHODS IN LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF N-ACETYLCYSTEINE IN PLASMA

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SUMMARY

Three electrochemical detection systems for the liquid chromatography of N-acetylcysteine in plasma have been compared: a dropping-mercury electrode detector, an amalgamated-gold electrode detector and a system in which iodine is generated electrochemically in the column effluent. The last detector approaches the reproducibility of the first (R.S.D. = 2%) and is as sensitive as the second, with a detection limit of 0.1 μg/ml plasma.

INTRODUCTION

In a study[1] on the treatment of amyotrophic lateral sclerosis patients with N-acetylcysteine (NAC), a reliable method to measure plasma concentrations of NAC was needed. Liquid chromatography with electrochemical detection (LC–ED) is now often used for the determination of thiol compounds in body fluids. Mercury-based electrodes are preferred, since thiols are oxidized very easily on mercury, due to the formation of mercury compounds[2,3] according to eqns. 1 and 2:

$$RSH + Hg \rightarrow RSHg + H^+ + e^- \quad (1)$$

$$2\ RSHg \rightarrow (RS)_2Hg + Hg \quad (2)$$

Therefore, thiols can be detected very selectively at a low detection potential in LC–ED with a mercury-based electrode[4,5]. We have tested two mercury-based detectors for the measurement of NAC in plasma after separation by reversed-phase liquid chromatography: a dropping-mercury electrode (DME) flow-through detector and an amalgamated-gold electrode [Hg(Au)] solid-state detector.

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Another method that we have tested is based on the homogeneous oxidation of NAC. Thiols can be oxidized by iodine to form disulphides:

$$RSH + 1/2\ I_2 \rightarrow 1/2\ RSSR + H^+ + I^- \quad (3)$$

The stoichiometry of this reaction is not always certain, especially since thiols with a free β-carbonyl group (as in the case of NAC) are known to be prone to overoxidation to sulphinic and ultimately sulphonic acids[6,7]:

$$RSH + 3\ I_2 + 3\ H_2O \rightarrow RSO_3^- + 7\ H^+ + 6\ I^- \quad (4)$$

We have made use of this oxidation to explore further an electrochemical detection system developed previously in our department[8]. The general concept of this system is the on-line, post-column electrochemical production of an oxidizing reagent from a precursor present in the mobile phase, and the amperometric measurement of any loss of reagent by a reaction with analytes in the column eluent. The eluent flows through the anode compartment of the production cell, then through a reaction coil and the amperometric detector, and back through the cathode compartment of the production cell. A previous application involved the production of bromine for the detection of thioethers in body fluids[9]. Since iodine has a lower standard redox potential than bromine (+0.53 V instead of +1.09 V in aqueous solutions), an improved selectivity compared to the bromine system is expected.

We have compared the performance of the iodine production method with that of the direct electrochemical techniques mentioned above.

EXPERIMENTAL

Apparatus

Voltammograms were recorded with a dropping-mercury electrode and with a gold rotating-disk (diameter 6 mm) electrode, driven by a servomotor with tachogenerator. The detectors for high-performance liquid chromatography (HPLC) were a fast-dropping (drop time 20–50 ms) mercury electrode flow-through cell, described in the literature[10], and a Metrohm (Herisau, Switzerland) 1096/2 cell with a gold working electrode (diameter 3 mm). Gold electrodes were amalgamated by rinsing them with 1 *M* nitric acid and methanol, and after drying, applying a drop of mercury on the surface for 5–10 min. The excess of mercury was removed with tissue paper. The mercury film was renewed every two or three days.

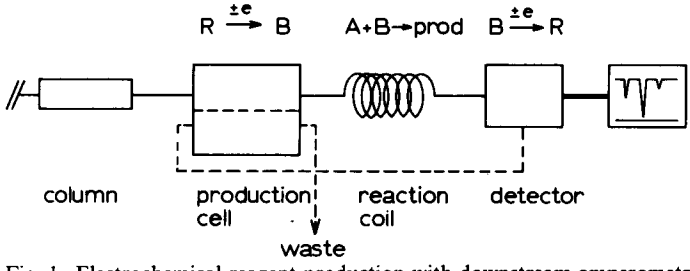

Fig. 1. Electrochemical reagent production with downstream amperometric detection.

The third detection system consisted of two electrochemical cells. The first, *i.e.*, the coulometric production cell was made in our workshop; we have published its characteristics and performance previously[8]. The second, *i.e.*, the amperometric detector cell, was a Metrohm 1096/2 cell with a 4-mm diameter platinum working electrode. The two cells were connected by a reaction capillary with a 6-s hold-up time (Fig. 1).

All potentials were measured against Ag/AgCl/1 *M* LiCl reference electrodes in methanol–water (1:1). A PAR 174 (Princeton Applied Research, Princeton, NJ, U.S.A.) and a laboratory-built potentiostat/amplifier were used. The constant-current source used for the iodine-generation system was also built in our laboratory.

The chromatographic equipment consisted of a Perkin-Elmer 601 pump delivering a flow-rate of 2 ml/min, a Rheodyne 7125 injection valve with 20- and 100-μl loops, and a 250 × 4.6 mm I.D. column, laboratory-packed with 10 μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.).

Reagents and solutions

NAC was U.S.P. XX quality, dithiotreitol (DTT) was high purity quality. Solutions were prepared with doubly distilled, demineralized water and Baker-grade methanol (Baker, Deventer, The Netherlands). Other chemicals used were of analytical-grade purity.

Standard NAC solutions (5–20 μg/ml) were prepared shortly before use from concentrated stock solutions (5%, w/v), which were stored at +4°C. Routinely, 50 μg/ml DTT were added to the standard solutions to keep NAC in the reduced form. Human pooled plasma samples (1 ml) were spiked with varying amounts of NAC and, after an incubation period (1 h, 32°C), with DTT. A second incubation period (1 h, 32°C) was applied to allow DTT to reduce all of the free oxidized NAC and to destroy the NAC–protein bonds[11]. The amount of DTT needed to accomplish this was determined experimentally by increasing the amount of DTT added until no further rise of the NAC-response was measured. A 200-μl volume acetonitrile–70% perchloric acid (4:1) was added to precipitate the proteins. Separation of the supernatant was achieved by centrifuging for 10 min in a table-top centrifuge. The supernatant was stored at −18°C.

Separation of NAC and DTT was achieved by HPLC with an aqueous phosphate buffer (0.05 *M*, pH 2.3) containing 5% methanol as the mobile phase. For the iodine-generation, 1 m*M* potassium iodide was added to the mobile phase.

RESULTS AND DISCUSSION

Voltammetry

The polarogram of NAC (Fig. 2a) in an aqueous phosphate buffer with 5% (v/v) methanol (pH 2.3) showed a diffusion-controlled anodic wave at −0.13 V *vs.* Ag/AgCl, a prewave at −0.27 V, caused by the adsorption of the mercury compound produced on the drop surface, and, for high NAC concentrations, a second wave at a positive potential. With the rotating Hg(Au) electrode, only a diffusion wave with $E_{1/2} = -0.12$ V was observed, and some irregularities at potentials above +0.25 V (Fig. 2b). The voltammograms suggest detection potentials between −0.05 and +0.20 V for the DME detector and between 0 and +0.25 V for the Hg(Au) detector.

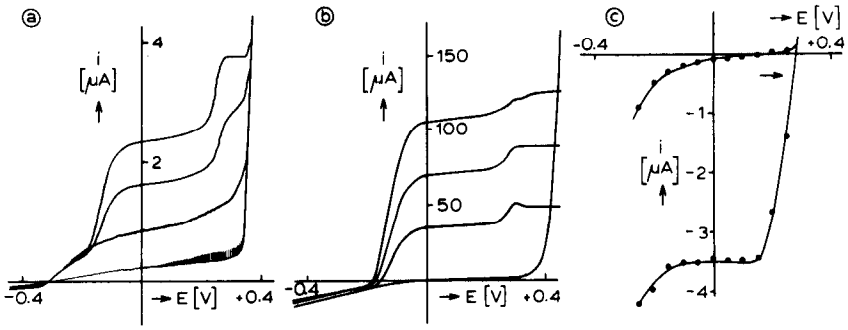

Fig. 2. Voltammetry: (a) DME polarograms of 0, 3, 6 and $9 \cdot 10^{-4}$ *M* NAC in mobile phase solution. (b) voltammograms with a rotating (20 rps) amalgamated-gold electrode of 0, 3, 6, and $9 \cdot 10^{-4}$ *M* NAC; (c) hydrodynamic voltammogram of the reduction of electrochemically generated iodine. Generating current, 0 and 100 μA.

A hydrodynamic voltammogram was measured for the reduction of electrochemically generated iodine (Fig. 2c). Potentials between −0.15 and +0.20 V could be used for amperometric measurements on the diffusion plateau of iodine.

Sample preparation

Our interest was in the total amount of biologically available NAC in plasma, *i.e.*, including the oxidized and protein-bound NAC. Therefore, as was described above, various amounts of DTT were added to the plasma before precipitation of the proteins. It was found that 2 mg DTT/ml plasma was sufficient to convert all NAC in spiked samples to the free, reduced form. The recovery of added NAC from plasma samples was 101 ± 8% (n = 12). The percentage recovery did not depend significantly on the amount added (5–20 μg/ml), nor on the detector used. For actual samples from patients who had been treated with NAC, 6 mg DTT/ml plasma was needed before a maximum in the amount of reduced NAC measured was reached. NAC concentrations in patient plasmas after treatment ranged from 10 to 75 μg/ml. To study the species of NAC in patient samples, experiments were carried out in which DTT was added after deproteination instead of before. In these experiments, no reduced NAC was found. It may be concluded that, in the patient samples, practically all NAC is bound to proteins.

Detector comparison

The DME detector was operated at an electrode potential of 0 V. This detection potential was a compromise: at more negative potentials an increasing interference of the cathodic oxygen peak was observed, while at more positive potentials the capacitive current increased, leading to higher noise levels and a stronger interference of negative peaks arising from double-layer capacity depression by bulk components of the plasma samples. A typical chromatogram of a plasma sample spiked with NAC and DTT is shown in Fig. 3a.

Some data on the performance of the DME detector are given in Table I. The main advantage of this detector is the excellent reproducibility. Even day-to-day variance was within 1% for standard solutions. Disadvantages are the relatively low

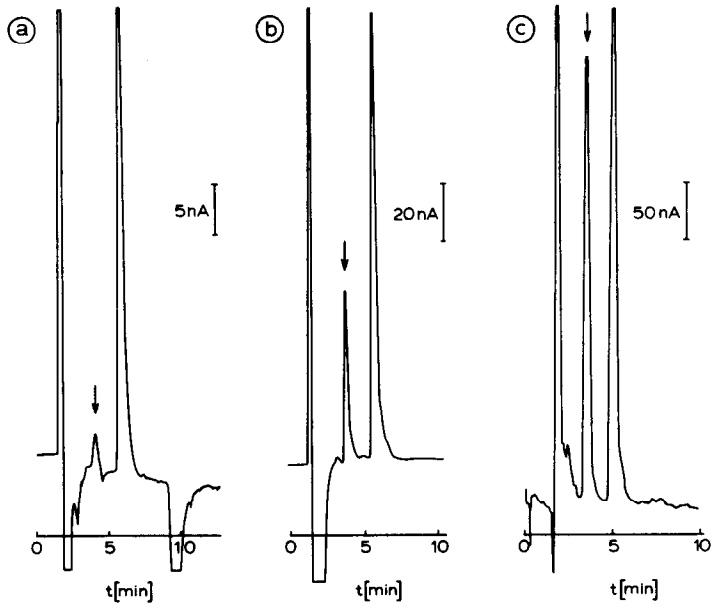

Fig. 3. Chromatograms of a plasma sample, spiked with NAC (5 μg/ml) and DTT. Detection systems: (a) DME; (b) Hg(Au); (c) I_2-generation. Column, 250 × 4.6 mm I.D. LiChrosorb RP-18, 10 μm; flow-rate, 2 ml/min; mobile phase, 5% methanol in 0.05 *M* phosphate buffer, pH 2.3; in (c): 1 m*M* potassium iodide added.

sensitivity, which makes a large injection volume (100 μl) necessary, and the practical difficulties in operating this detector.

A chromatogram obtained with the Hg(Au) detector is shown in Fig. 3b. This detector was operated at +0.2 V, so that less interference of the oxygen dissolved in the sample was encountered. The sensitivity for NAC in standard solutions is two orders of magnitude better than with the DME detector. With plasma samples, chemical noise is more important than instrumental noise. Still, the lowest detectable serum

TABLE I

DETECTOR PERFORMANCE

S/N = signal-to-noise ratio.

Detector	*DME*	*Hg(Au)*	*I_2-generation*
Detection characteristics	E = 0.0 V	E = +0.2 V	i_G = 50 μA
Typical sensitivity for NAC	10 nA/μg inj.	0.5 μA/μg inj.	5 μA/μg inj.
Noise	0.2 nA	0.1 nA	5 nA
Detection limit (S/N = 2)	40 ng	0.4 ng	2 ng
Lowest NAC concentration in plasma	1 μg/ml	0.2 μg/ml	0.1 μg/ml
Peak-height reproducibility			
For standards	±1%	± 7%	±2%
For plasma	±6%	±11%	±2%

concentration of NAC is improved by a factor of 5 compared with the DME detector, while the injection volume could be reduced to 20 μl (see Table I).

The main problem with the Hg(Au) detector is the poor reproducibility. Even for consecutive injections of standard solutions, the relative peak-height variance was 7%. The electrode reponse could change from day to day by a factor of 2, possibly by changes in the thickness of the mercury film. Frequent recalibration was therefore necessary. When plasma samples were analysed, the electrode response to NAC could sometimes drop dramatically, probably by adsorption of plasma components on the electrode surface. We found that this effect could be eliminated by applying a potential of -1.2 V for 1 min. Therefore, these potential pulses were applied routinely after each plasma analysis.

The iodine-reaction system was operated with generating currents from 20 to 100 μA and a detection potential of $+0.15$ V. With this system, the relative noise level is higher than in the direct amperometric mode, and, subsequently, the detection limit with standard solutions is higher. The noise level is proportional to the baseline current and therefore dependent on the height of the generating current. By lowering the generating current, the detection limit can be improved but at the same time the upper limit of linear response will then be decreased. With a generating current of 50 μA, no deviations from linearity were observed in the range up to 200 ng injected.

From the areas of the NAC peaks obtained, it was calculated that 1 mol NAC reacts with 2.9 ± 0.1 mol I_2. It may be concluded that, under the conditions employed, the reaction between NAC and iodine proceeds as given by eqn. 4.

In flow-injection experiments it was found that the contribution of the production cell and reaction coil to band broadening was 1.4 ± 0.5 s^2. In chromatograms, no differences in peak widths were observed compared with the other two detectors.

Injection of deproteinized plasma samples yielded very clean chromatograms with the iodine-reaction system (see Fig. 3c). Despite the higher instrument noise, the lowest detectable plasma concentration was even lower than with the Hg(Au) detector. In fact, it was only with the iodine-reaction detector that the NAC concentration in the blank, pooled human plasma could be measured (0.2 μg/ml).

The reproducibility of the iodine-production detector was very good (see Table I). Only the first two or three standard injections in a series of experiments tended to result in low responses. We blamed this on the presence of small amounts of iodine, formed in the mobile phase and accumulated on the column during the time when no oxidizable compounds were injected. Indeed, this phenomenon could be suppressed by adding a small amount (10^{-6} *M*) of thiosulphate to the mobile phase.

CONCLUSIONS

Each of the three detection modes studied for thiol compounds has its advantages and disadvantages. The DME detector lacks the sensitivity for real trace analysis in body fluids. However, when more concentrated solutions must be analysed, its excellent (day-to-day) reproducibility is a distinct advantage. For instance, we have used the DME detector to our satisfaction in a study on the stability of NAC in infusion solutions. The Hg(Au) detector has a simple design and a high sensitivity. However, in our opinion, the instability of the electrode response will remain a draw-

back for routine analysis. Though some authors pay attention to this problem, we feel that it is somewhat understated in the literature. For the iodine-reaction detection system, some additional apparatus is needed. However, both the production cell and the constant current scource can be manufactured at relatively low cost. Its sensitivity is at least as good as that of the Hg(Au) detector. The main advantage of this detection mode is that the troublesome heterogeneous electrode processes are replaced by much better controllable, homogeneous oxidation reactions.

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