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The determination of thiomalate in physiological fluids by high-performance liquid chromatography and electrochemical detection

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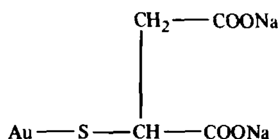
Abstract: Methods are described for the determination of free thiomalate in the plasma and urine of patients receiving the anti-rheumatic drug sodium aurothiomalate. Thiomalate is separated by reversed-phase chromatography and detected using a gold electrochemical cell. Plasma analyses require maximal sensitivity while urine estimations require selectivity rather than sensitivity: different phosphate buffer–methanol eluents and electrode potentials are used. On-column sensitivity for thiomalate is 40 fmol injected.

Keywords: *Thiomalate; sodium aurothiomalate; reversed-phase chromatography; electrochemical detection; rheumatoid arthritis.*

Introduction

Sodium aurothiomalate is one of several sulphur-containing drugs used in the treatment of progressive rheumatoid arthritis; others include penicillamine and levamisole [1, 2]. In animal studies sodium aurothiomalate has been shown to dissociate immediately following intramuscular injection, presumably to produce protein-bound gold and free thiomalate [3]. While the gold moiety has been extensively studied [4, 5], little is known of the pharmacokinetics of thiomalate. Since both the efficacy and toxicity of sodium aurothiomalate may be dose-related, and studies to date have found no correlation with gold levels in physiological fluids [6, 7], an assay for thiomalate might be of value in optimizing the use of this agent. Such a method has therefore been developed using reversed-phase liquid chromatography followed by electrochemical detection; a similar principle has been used for the determination of penicillamine [8] and captopril [9].



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Experimental

Materials

The chromatographic system comprised an ACS 300/01 pump (Applied Chromatography Systems, Luton, UK) and an EDT electrochemical detector LCA 15 (E.D.T. Research, Acton, London, UK) equipped with a LC-19 gold cell (Bioanalytical Systems, West Lafayette, Indiana, USA). The cell was maintained at +0.60 to +0.80 V versus an Ag/AgCl reference electrode. A 150 mm length and 5 mm internal diameter column packed with 3 μm ODS-Hypersil (Shandon, Runcorn, UK) was used with a 7125 valve injector equipped with a 20 μl loop (Rheodyne, Cotati, California, USA). The column was connected to the cell by 15 cm of 0.15 mm i.d. ptfe tubing. Output from the cell was recorded on a Servoscribe recorder. Thiomalic acid was obtained from the Sigma London Chemical Company (Poole, UK); potassium dihydrogen phosphate AR, disodium hydrogen phosphate AR, disodium EDTA AR, sulphosalicylic acid AR and orthophosphoric acid AR from BDH Chemicals (Poole, UK), and methanol from Rathburn Chemicals (Walkerburn, Scotland, UK). Water was distilled and then deionized immediately before use.

The eluent consisted of aqueous phosphate buffer (Na_2HPO_4 80 mmol/l and KH_2PO_4 20 mmol/l) adjusted to either pH 2 or pH 1.3 with H_3PO_4. For plasma samples 8% methanol was optimal; for urine samples only 1% methanol was required. All eluents were filtered before use and continuously degassed with oxygen-free nitrogen. The mobile phase flow rate was 1.2 ml/min.

Samples

Stock thiomalate standard (1 mmol/l) was prepared in 100 mmol/l H_3PO_4. Urine from patients receiving sodium aurothiomalate (Myocrisin; May & Baker, Dagenham, UK) was collected directly into containers holding 1 ml of 6 mol/l HCl per 100 ml of urine; aliquots were then frozen at −20°C until assay. Whole blood was collected into tubes containing Na_2EDTA (final concentration 5 mmol/l) and centrifuged for 5 min. Plasma proteins were precipitated by the addition of 100 μl of sulphosalicylic acid (2 mol/l) to 1 ml of plasma. After a further 5 min centrifugation the supernatant was rapidly frozen and then stored at −20°C until analysis. Immediately before chromatography the sample was centrifuged for 5 min: 20 μl was then injected onto the column.

Results are presented as mean ± 1 SD.

Results

Thiomalate could be directly oxidized at a glassy carbon, gold or mercury electrode. Figure 1 shows the hydrodynamic voltammogram for each electrode demonstrating an optimum working potential for gold of +0.80 V. The gold electrode was considered to be most satisfactory for routine use and was employed in all subsequent work (see Discussion). Figure 2 shows the effect of buffer pH on the chromatography. Both retention time and peak height were maximal below pH 2. Double peaks were obtained at pH 4 or above, possibly due to different ionic forms of thiomalate being present; thus all subsequent studies were performed at pH 2 or below. Using these conditions a plate number of 48 000/m was obtained.

Figure 3 shows standard chromatograms obtained following the injection of 2 and 20 pmol of thiomalate under the conditions described above. Maximum sensitivity was 40 fmol injected (S/N=2) on to the column (equivalent to 2 nmol/l). Reproducibility of the

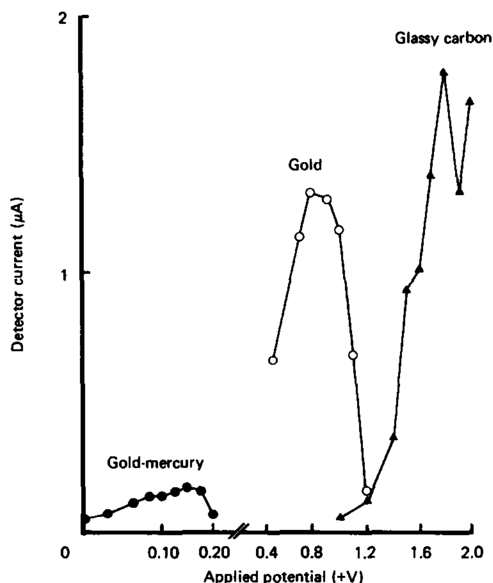

Figure 1
Hydrodynamic voltammograms for thiomalate using gold/mercury (●—●), gold (○—○) and glassy carbon (▲—▲) electrodes. 2 nmol of thiomalate was injected on each occasion.

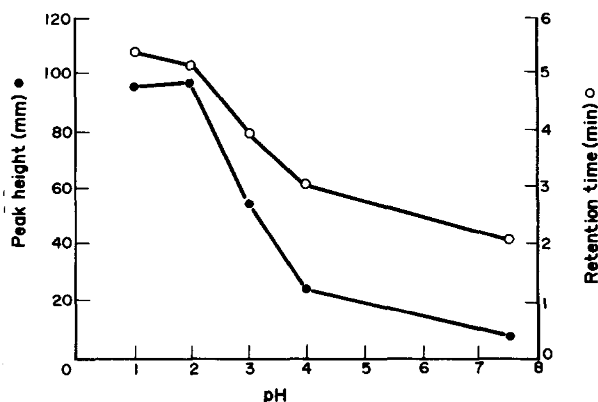

Figure 2
The effect of buffer pH on chromatography showing peak height (●) and retention time (○).

peak height following repeated injection of 200 pmol of standard was 111.5 + 2.3 mm (n = 10). The detector response was linear with increasing amounts of thiomalate over the range 0–2 nmol injected (detector response (nA) = 0.06040 × thiomalate injected (pmol) + 0.006716; standard error of slope = 0.00137, $n = 9$, $r = 0.9982$, $p < 0.001$). Stock standard solutions (1 mmol/l) were stable for at least one month when stored at 4°C. Working standards (100 μmol/l–100 nmol/l) were prepared daily.

Plasma samples

Figure 4 shows thiomalate in the plasma of a patient who had received 20 mg of sodium aurothiomalate by intramuscular injection 60 min earlier. The concentration of thiomalate was 580 nmol/l. The thiomalate peak co-chromatographed with authentic thiomalate added to the same plasma (Fig. 4). Recovery of thiomalate added to plasma and immediately deproteinized was 71.7 ± 3.8% (n = 5). Overall reproducibility in plasma was 11.1% for 20 pmol injected on to the column (n = 8).

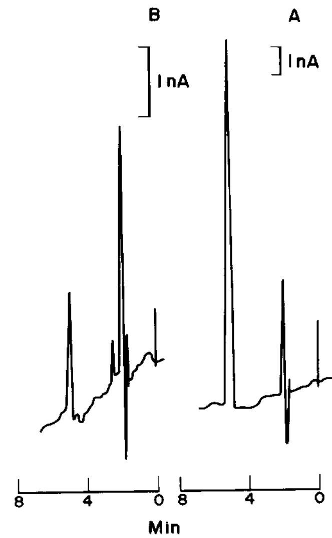

Figure 3
Chromatograms of (A) 20 pmol and (B) 2 pmol of thiomalate injected as described in text.

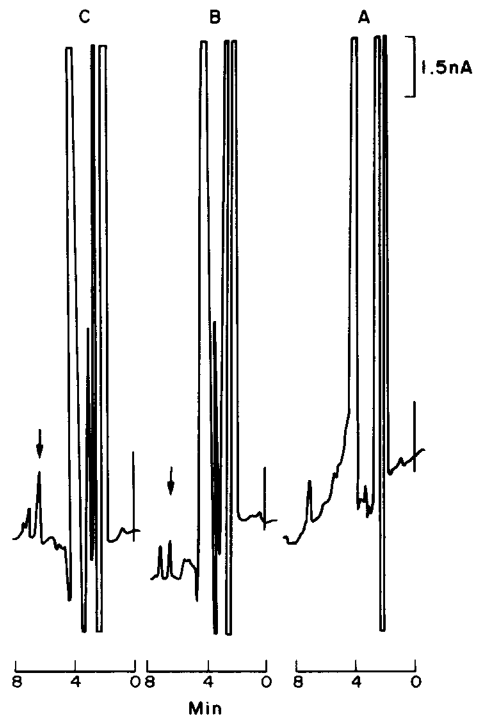

Figure 4
Chromatograms showing detection of thiomalate in plasma extracts, (A) before injection, (B) 60 min following injection of 20 mg sodium aurothiomalate, (C) as (B) but with added thiomalate. Arrows indicate thiomalate peaks.

Urine samples

Urine contains several endogenous compounds capable of interfering with the estimation of thiomalate. Optimum resolution was obtained by reducing the methanol concentration to 1% and by using a working potential of +0.60 V (Fig. 5): in view of the relatively high concentrations of thiomalate in urine, maximum sensitivity was not

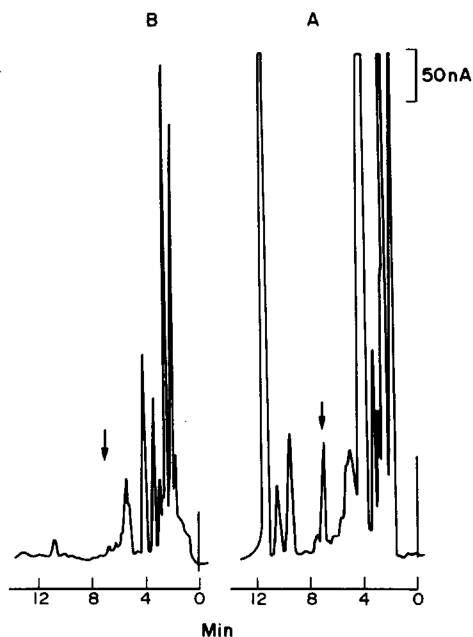

Figure 5
Effect of detector voltage on elution profile of blank urine. (A) + 0.80 V, (B) + 0.60 V v Ag/AgCl. Arrows show expected elution time for thiomalate.

necessary. Figure 6 shows thiomalate in the urine of a patient who had received 20 mg of sodium aurothiomalate by intramuscular injection 1 h earlier. Under these conditions recovery of thiomalate from urine was 94.5 ± 3.5% (n = 6) and the reproducibility was 3.7% for 500 pmol injected (n = 8).

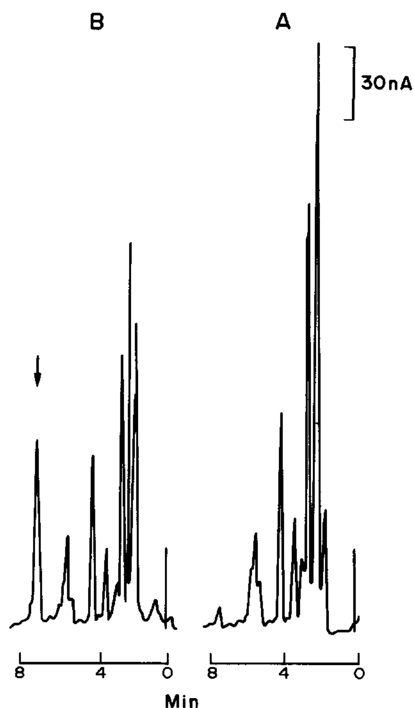

Figure 6
Chromatograms showing detection of thiomalate in urine, (A) before injection, and (B) 60 min following injection of 20 mg sodium aurothiomalate. Arrow indicates thiomalate peak.

Discussion

Many thiol-based drugs have been shown to be effective anti-rheumatic agents. These include sodium aurothiomalate [10], D-penicillamine [1], levamisole [2], 5-thiopyridoxine [11] and auranofin [12]. It is therefore tempting to postulate that the activity of these agents results from a specific action of the sulphydryl group.

Thiol compounds have always proved difficult to quantitate in physiological fluids both because of their inherent instability and the presence of endogenous thiols. The combination of high-performance liquid chromatography with electrochemical detection has recently been shown to be a simple and rapid means for the detection of D-penicillamine and captopril in plasma and urine [8, 9]. The present method, similar in principle, has greater sensitivity than either of these and is reproducible and simple to perform.

Although thiomalic acid could also be oxidized directly at a gold/mercury or glassy carbon electrode, each has its disadvantages. At the optimum working potential required with the glassy carbon electrode (+1.8 V) many other substances in plasma interfered with chromatography and there was a considerable increase in noise, limiting sensitivity. Whilst the gold/mercury electrode has been shown to be highly specific for thiols, it proved much less stable than the gold and is also less sensitive (Fig. 1). Frequent loss of sensitivity occurred requiring re-formation of the mercury/gold amalgam. Although the precise reaction occurring at the gold electrode is unknown, it proved to be the most satisfactory detector.

Jellum, Munthe and colleagues used gas chromatography and mass spectrometry to detect, but not quantitate, free thiomalate in the urine of patients with rheumatoid arthritis who had received 50 mg of sodium aurothiomalate [13, 14]. They were, however, unable to find thiomalate in serum or synovial fluid. We have detected and quantitated free thiomalate in the plasma and urine of patients receiving an initial dose of only 20 mg of sodium aurothiomalate. This technique will thus allow investigation of the pharmacodynamics of thiomalate in man.

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