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DETERMINATION OF DISULFIRAM AND TWO OF ITS METABOLITES IN URINE BY REVERSED-PHASE LIQUID CHROMATOGRAPHY AND SPECTROPHOTOMETRIC DETECTION AFTER POST-COLUMN COMPLEXATION

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SUMMARY

A selective method was developed for the determination of disulfiram and two of its metabolites, diethyldithiocarbamate (DTC) and copper(II) diethyldithiocarbamate [$Cu(DTC)_2$], in complex (biological) samples by reversed-phase liquid chromatography (RP-LC) with post-column derivatization. In the first step, DTC is converted into lead(II) diethyldithiocarbamate [$Pb(DTC)_2$] by adding lead(II) acetate. Disulfiram, $Pb(DTC)_2$ and $Cu(DTC)_2$ can be easily pre-concentrated on C_{18}-bonded silica. After separation by isocratic RP-LC, derivatization takes place in two solid state post-column reactors packed with metallic copper and copper(II) phosphate. Disulfiram reacts with metallic copper to form $Cu(DTC)_2$. The same product is obtained by the ligand-exchange reaction between $Pb(DTC)_2$ and copper(II) phosphate. $Cu(DTC)_2$ can be detected selectively at 435 nm with good sensitivity (molar absorptivity, $\epsilon = 13\,000$). The derivatization reactions proceed rapidly and quantitatively, which was confirmed by comparison of absorption spectra. The applicability of this method is demonstrated for undiluted urine samples which, apart from the addition of lead(II) acetate and pre-concentration on C_{18}-bonded silica, require no clean-up procedure.

INTRODUCTION

Disulfiram (DSF) (tetraethylthiuram disulphide; Antabuse) has been used as a drug against alcohol abuse for about 35 years [1]. Despite its frequent use, few data are known about its pharmacokinetics and metabolism in humans. The first and rapid metabolic reaction after the administration of disulfiram is its reduction to diethyldithiocarbamate (DTC) [1]. DTC is more stable in human blood; Cobby et al. [1] reported a half-life of 70 min for DTC whereas DSF was reduced completely within 4 min. Further metabolism of DTC (see Fig. 1) can lead to the formation of its methyl ester [2,3], carbon disulphide [2] or copper

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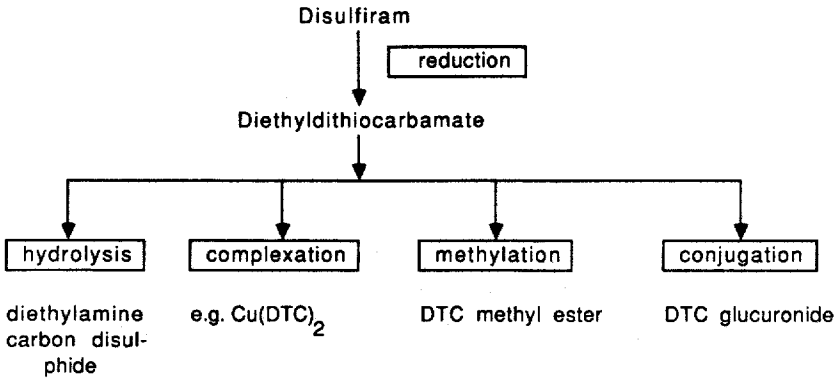

Fig. 1. Metabolism of disulfiram (DTC = diethyldithiocarbamate).

bis(diethyldithiocarbamate) [4,5]. The last compound is formed by complexation of DTC with protein-bound copper(II) ions present in plasma. Urinary metabolites are the S-glucuronide of DTC and inorganic sulphate [6] (see Fig. 1).

Modern methods for the determination of disulfiram and its metabolites are mainly based on gas chromatography (GC) [1,7] or liquid chromatography (LC) [4,8,9]. GC methods require tedious sample pre-treatment steps and therefore they are not suitable for the monitoring of rapid metabolic reactions. LC allows the rapid determination of DSF and its metabolites in plasma with pre-concentration on a C_{18}-bonded phase, separation on a C_{18} column and detection at 254 nm, as demonstrated by Johansson [4]. DSF, DTC (after ethylation), DTC methyl ester, copper(II) diethyldithiocarbamate [$Cu(DTC)_2$] and carbon disulphide (after conversion into dimethyldithiocarbamate ethyl ester) were also determined.

The non-selective UV detection, however, still is a major drawback because other non-polar, UV-absorbing compounds may seriously interfere with the determination of DSF and its metabolites in biological samples (see Fig. 2A). In order to overcome this problem we have investigated the possibility of enhancing the selectivity by applying the well known reaction of dithiocarbamates with copper(II) as a post-column derivatization step with subsequent detection at 435 nm. Fig. 2B shows the effect of higher wavelength on the selectivity for the analysis of urine. The first results on the use of a copper post-column reactor for the derivatization of thiram and DSF were published recently [10]. In this work the method has been extended to the determination of two DSF metabolites, DTC and $Cu(DTC)_2$, in urine and involves, in addition to metallic copper, copper(II) phosphate as a derivatization reagent.

EXPERIMENTAL

Chemicals

DSF was purchased from Fluka (Buchs, Switzerland) and was of 98% purity. Sodium N,N-diethyldithiocarbamate [Na(DTC)] and EDTA were supplied by EGA Chemie (Steinheim, F.R.G.). All other organic chemicals were of analyti-

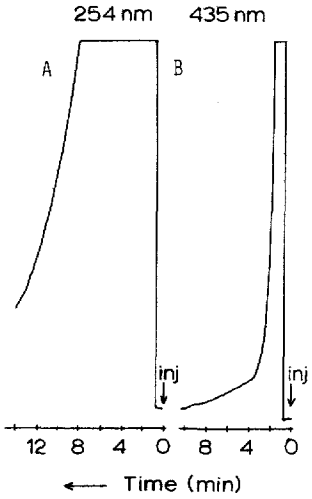

Fig. 2. Chromatogram of a blank urine sample (5 ml of urine pre-concentrated) recorded at (A) 254 nm (0.02 a.u.f.s.) without a post-column reactor and (B) at 435 nm (0.02 a.u.f.s.) with a post-column reactor. Conditions: analytical column, 100 mm × 4.6 nm I.D. packed with 5-μm Hypersil ODS; pre-column, 2.0 mm × 4.6 mm I.D. packed with 5-μm LiChrosorb RP-18; eluent, acetonitrile–acetate buffer (10 m*M*, pH 5.3) (70:30); flow-rate, 1.0 ml/min; post-column reactors, (1) 2.0 mm × 4.6 mm I.D. packed with metallic copper and (2) 4.0 mm × 4.6 mm I.D. packed with copper(II) phosphate.

cal-reagent grade (Baker, Deventer, The Netherlands). Copper(II) sulphate, lead(II) acetate, potassium citrate, potassium hydrogen phosphate and copper(I) chloride were Baker-analyzed reagents. Sodium borohydride was of 99% purity (Baker).

$Cu(DTC)_2$ and lead(II) diethyldithiocarbamate [$Pb(DTC)_2$] were prepared by adding a 1 m*M* solution of copper(II) sulphate and lead(II) acetate, respectively, to a 2 m*M* aqueous solution of Na(DTC) in 10 m*M* phosphate buffer (pH 6.8). All experiments were carried out with fresh solutions to avoid precipitation of the metal dithiocarbamates.

Instrumentation

The LC system (Fig. 3) consisted of a Kontron (Zürich, Switzerland) LC pump and a 200 mm × 2.1 mm I.D. or 100 mm × 4.6 mm I.D. stainless-steel column packed with 5-μm Hypersil ODS (Shandon Southern, Cheshire, U.K.). A Hewlett-Packard (Waldbronn, F.R.G.) 1040 diode-array detector was used at a detection wavelength of 435 nm. Trace enrichment was carried out on a laboratory-made [11] 4.0 mm × 2.1 mm I.D. pre-column, which was manually packed with a slurry of 5-μm Hypersil ODS in methanol using a syringe. An Altex (Berkeley, CA, U.S.A.) pre-concentration pump was used at a flow-rate of 1.0 ml/min. For the post-column reactor the same type of pre-columns of length 2.0 and 4.0 mm and I.D. 4.6 mm were used. Acetonitrile–aqueous acetate buffer (10 m*M*, pH 5.3) was used as the LC mobile phase; it was degassed ultrasonically under vacuum for 20 min.

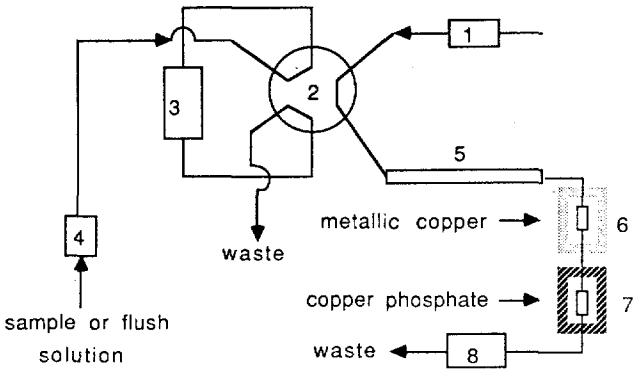

Fig. 3. Schematic diagram of the apparatus. 1, LC pump; 2, injection valve; 3, pre-column; 4, pre-concentration pump; 5, analytical column; 6, post-column reactor packed with metallic copper; 7, post-column reactor packed with copper(II) phosphate; 8, UV-visible absorbance detector.

Pre-treatment of urine samples

Urine samples were filtered through Millipore (Bedford, MA, U.S.A.) filters after their pH had been adjusted to 6.8 using 10 m*M* phosphate buffer. They were spiked with DSF and, subsequently, 0.5 ml of a 0.1 *M* aqueous lead(II) acetate solution was added. In order to avoid precipitation of the water-insoluble metal complexes the sample was directly pumped to the pre-column. The pre-concentration system had previously been flushed with 5 ml of acetonitrile and then an aqueous EDTA–potassium citrate solution (1:1, 10 m*M*) in order to remove organic compounds and metal ions.

Post-column derivatization system

Derivatization was carried out on two solid-state post-column reactors. The first reactor consisted of a 2.0 mm×4.6 mm I.D. column packed with metallic copper, which was prepared by reduction of copper(I) chloride with sodium borohydride (for further details, see ref. 10). The second reactor was a 4.0 mm×4.6 mm I.D. column filled with copper(II) phosphate, which was prepared by mixing equal volumes of a 0.1 *M* solution of potassium hydrogenphosphate and a 0.1 *M* solution of copper(II) sulphate. The precipitated copper(II) phosphate was washed twice with doubly distilled water and methanol. After treating the copper(II) phosphate suspension in methanol ultrasonically for 20 min, it was dried on tissue paper and pressed as densely as possible into the column using a microspatula.

RESULTS AND DISCUSSION

Separation and derivatization system

The reaction of dithiocarbamates and thiuram disulphides with metallic copper and copper(I) or copper(II) compounds has often been used in spectrophotometric methods for this class of compounds [12]. The intensely coloured $Cu(DTC)_2$ which is formed has an absorption maximum at 435 nm with a molar

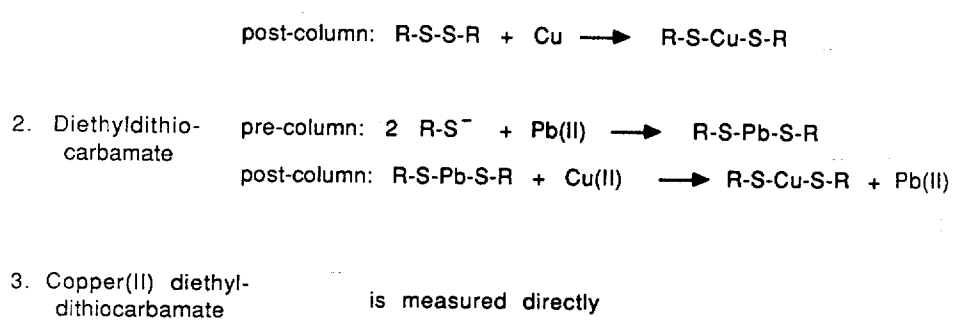

Fig. 4. Derivatization reactions [$R=(C_2H_5)_2NC(S)-$].

absorptivity (ϵ) of 13 000 [13], and therefore offers selective and sensitive detection. Recently we demonstrated the application of this reaction for the post-column derivatization of thiram and DSF [10]. Metallic copper was used as a complexation agent; the reaction is shown in Fig. 4.

In this work, the determination of both DSF and two of its metabolites, DTC and $Cu(DTC)_2$, was investigated. The last compound can be measured directly without derivatization whereas DTC, which is present as the anion at physiological pH values, has to undergo both pre- and post-column derivatization to render it suitable for reversed-phase LC with detection at 435 nm. Chromatography of this compound in the undissociated form is not possible because of the rapid breakdown of DTC at pH < 4. In order to increase its apolarity, DTC is usually methylated using methyl iodide [4,8,9]. A similar effect can be obtained by forming metal complexes which can be chromatographed on, e.g., C_{18}-bonded silica [14]. In this work, the reaction of DTC with lead(II) was chosen for this purpose because then post-column ligand exchange with copper(II) ions is possible (see Fig. 4). An additional advantage of this complexation reaction is the increased stabilization of DTC against hydrolysis and against complexation reactions with other metals present in the metallic parts of the LC system.

The pre-column reaction is performed by adding an excess of lead(II) acetate to the sample, which is buffered at pH 6.8 using a 10 m*M* phosphate buffer. $Pb(DTC)_2$ which is formed in this reaction is of low polarity and can easily be enriched on C_{18}-bonded silica [14]; DSF and $Cu(DTC)_2$ do not react with lead(II) ions. The three compounds can be separated on a C_{18} analytical column using acetonitrile–aqueous acetate buffer (65:35) as the mobile phase.

Attempts to use the metallic copper post-column reactor for the post-column derivatization of $Pb(DTC)_2$ in order to detect it at 435 nm were not successful, as $Pb(DTC)_2$ reacts only slowly with the metallic copper used for the derivatization of DSF. The latter reaction is a redox reaction which proceeds even in pure organic solvents such as chloroform [15]; $Pb(DTC)_2$, however, reacts via ligand exchange and therefore requires copper ions for a rapid reaction (see Fig. 4). Therefore, a copper(II) salt was selected with (i) a low stability constant and (ii) low solubility in the LC eluent. Copper(II) phosphate was found to fulfil

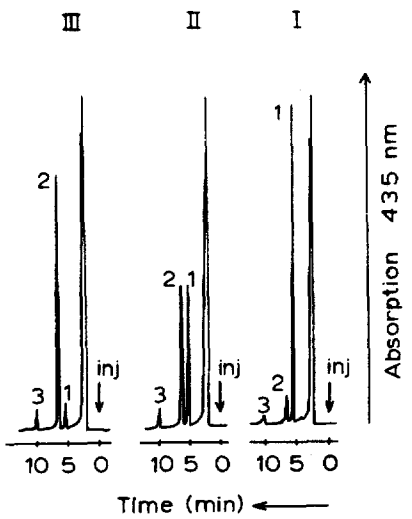

Fig. 5. Determination of disulfiram and metabolites in urine. Liquid chromatograms recorded (I) 1 min, (II) 20 min and (III) 40 min after addition of 500 ppb of disulfiram to a urine sample (1 ml pre-concentrated). Peaks: 1 = Disulfiram; 2 = DTC [as $Pb(DTC)_2$]; 3 = $Cu(DTC)_2$. Conditions: analytical column, 200 mm × 2.1 mm I.D. packed with 5-μm Hypersil ODS; eluent, acetonitrile–acetate buffer (pH 5.3, 10 m*M*) (65:35); flow-rate, 0.5 ml/min; detection wavelength, 435 nm (0.1 a.u.f.s.); post-column reactors, as in Fig. 2.

these conditions. It is nearly insoluble in water and acetonitrile (with the solubility decreasing at higher temperatures [16]) and has a low stability constant [17].

Post-column derivatization with copper(II) phosphate

When using copper(II) phosphate as a derivatization reagent in a solid-state post-column reactor, $Pb(DTC)_2$ was converted rapidly and completely into $Cu(DTC)_2$ as expected because of the large difference in complex stability {log β_2 [$Cu(DTC)_2$] = 21.8 [18] vs. log β_2 [$Pb(DTC)_2$] = 7.9 [17]}. Absorption spectra of the two peaks corresponding to the lead and copper complexes were recorded with a diode-array detector and found to be identical. At LC flow-rates relevant for 2.1 mm I.D. analytical columns (0.4–0.6 ml/min) complete conversion is obtained at room temperature with a reactor bed of 4.0 mm × 4.6 mm I.D. Under these conditions the response for $Pb(DTC)_2$ was linear over almost three orders of magnitude ($r = 0.999$) and a detection limit of 7 ng was obtained at a signal-to-noise ratio of 3:1. The R.S.D. was 1.9% ($n = 5$) at concentrations of 10 ppm.

Attempts to use copper(II) phosphate for the post-column derivatization of DSF were not successful, the conversion rates even at 70 °C and a flow-rate of 0.5 ml/min being only about 40%. Therefore, two post-column reactors were used in series, the first (2.0 mm × 4.6 mm I.D.) packed with metallic copper and the second (4.0 mm × 4.6 mm I.D.) with copper(II) phosphate. The band broadening caused by each reactor was about 2 s, but this did not adversely affect the sepa-

ration of the three compounds (see Fig. 5). Preliminary investigations have shown that mixing of the two reactants in one bed is possible, which may lead to a further reduction of the band broadening.

Determination of DSF, DTC and $Cu(DTC)_2$ in urine

Using the described method, DSF, DTC and $Cu(DTC)_2$ were determined simultaneously in urine. It should be noted that DSF will not be found in urine under actual conditions owing to its instability. In Fig. 5 the analysis of a urine sample spiked with 500 ppb of DSF is shown. A 1-ml urine sample was pre-concentrated on a 4.0 mm × 2.1 mm I.D. pre-column packed with C_{18}-bonded silica and was eluted with forward flushing to the analytical column. Chromatograms were recorded after 1, 20 and 40 min storage of the sample; they clearly show the rapid degradation of DSF and the increasing DTC concentration, as indicated by the $Pb(DTC)_2$ peak. The $Cu(DTC)_2$ peak probably arises from the reaction of DTC with copper ions present in urine. Apart from a large unretained peak at t_0 only analyte peaks are observed, which demonstrates the high selectivity of the method. The total analysis time including the trace enrichment step is about 15 min.

The breakthrough volume of all three analytes on C_{18}-bonded silica is higher than 50 ml. With trace enrichment of 5 ml, low ppb concentrations of DSF can still be detected in urine. At such low concentrations interferences from the metallic parts of the LC system have been observed, which may lead to ligand-exchange reactions of $Pb(DTC)_2$ with nickel(II), cobalt(II) or copper(II). This problem is common in methods for the determination of strongly complexing analytes. The effect was minimized by flushing the whole system with 5 ml of a 10 m*M* solution of EDTA–potassium citrate (1:1) and did not play a role in the urine analysis mentioned above.

CONCLUSIONS

Post-column derivatization with metallic copper and copper(II) phosphate allows the selective determination of DSF, DTC and $Cu(DTC)_2$ in a biological matrix with minimal sample pre-treatment. Both post-column reactors are easy to prepare and they possess long lifetimes, i.e., several days for the metallic copper-containing reactor and a few weeks for the copper(II) phosphate reactor. The low polarity of all analytes allows their pre-concentration on reversed-phase materials, which easily yields enrichment factors of over 500 with detection limits in the low ppb range. On-line sample handling renders the method suitable for pharmacokinetic studies of both DSF and its primary metabolite, diethyldithiocarbamate. The selectivity of the detection wavelength permits relatively short analysis times of about 15 min. The scope of the method may be extended to the determination of other metabolites such as DTC glucuronide or DTC methyl ester by introducing on-line pre-column hydrolysis with immobilized glucuronidase [19] or an ion exchanger [20], respectively.

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