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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POLAROGRAPHIC AND VOLTAMMETRIC ANODIC DETECTION: SIMULTANEOUS DETERMINATION OF ALLOPURINOL, OXIPURINOL AND URIC ACID IN BODY FLUIDS

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SUMMARY

Allopurinol, oxipurinol and uric acid have been determined in human serum and urine by liquid chromatography with electrochemical detection. In particular the use of a polarographic detector operating in the oxidative mode, whose principle of detection is based on the property of allopurinol, oxipurinol and uric acid to form insoluble anodic films on mercury, is described. The performance of such a detector is compared with that of a glassy carbon wall-jet detector. Different procedures for sample pretreatment have been evaluated.

INTRODUCTION

Allopurinol (1H-pyrazolo[3,4-*d*]pyrimidin-4-ol) and its major active metabolite oxipurinol (1H-pyrazolo[3,4-*d*]pyrimidin-4,6-diol) are powerful xanthine oxidase inhibitors [1] extensively used in the treatment of purine (and pyrimidine) metabolic disorders which lead to the formation of an excess of uric acid in humans.

Procedures capable of detecting simultaneously the primary drug (allopurinol), its metabolite (oxipurinol) and uric acid (effect) in body fluids are of considerable interest for pharmacokinetics and/or clinical studies. Several high-performance liquid chromatographic (HPLC) methods have been devised for the determination of allopurinol and oxipurinol in body fluids: reversed-phase HPLC [2], ion-exchange HPLC [3] and ion-exchange HPLC in combination with purification on Chelex-100 resin [4]. At the same time, several HPLC methods are available for the determination of uric acid separately [5—7] or simultaneously [8] with other pyrimidines and purines. Recently

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an HPLC method (with UV detection at 254 nm) for the simultaneous determination of allopurinol, oxipurinol and uric acid in human plasma has been reported by Nissen [9]. However, unidentified compounds were found to interfere with allopurinol and/or oxipurinol in six of eleven blank plasma samples taken from different subjects. Moreover, since no sample pretreatment was adopted the renewal of the precolumn after 50—70 applications was necessary because of clogging.

We have recently undertaken [10] an electrochemical study on the anodic behaviour of allopurinol and oxipurinol on mercury and glassy carbon electrodes. Both compounds (and uric acid) can be electrooxidized on glassy carbon; at mercury electrodes they give rise to anodic waves due to the formation of sparingly soluble compounds with mercury. This latter peculiarity is of a certain interest from an analytical point of view since it permits the use of highly sensitive electrochemical techniques such as differential pulse cathodic stripping voltametry (DPCSV). However, in spite of the great sensitivity of DPCSV (detection limits typically in the order of 10^{-9} mol/l), the direct determination of the investigated drugs in complex matrices such as body fluids is not possible owing to severe interference effects. Evidently electrochemical methods of analysis could be employed only if coupled to a separating technique such as liquid chromatography.

In this paper we wish to report an HPLC method for the simultaneous determination of allopurinol, oxipurinol and uric acid in both human serum and urine, which makes use of a polarographic (dropping mercury electrode) detector operating in the oxidative mode; the detection principle is based on the peculiar anodic behaviour shown by these compounds (e.g. the formation of an insoluble anodic film). The use of a glassy carbon electrochemical detector (wall jet type) is also described and the relative merits of both detectors are discussed. Different procedures for sample pretreatment have been also evaluated.

EXPERIMENTAL

Apparatus

A Perkin Elmer Model 3B pump module equipped with a Rheodyne 7125 injector and a reversed-phase column (Perkin Elmer RP-8, 10 μm, 250 × 4.6 mm) was used as the chromatographic system. When necessary a Brownlee RP-GU guard cartridge (30 × 4.6 mm), fitted into a Brownlee MPLC holder, was used to protect the analytical column.

A home-made pulse dampener (consisting of an air damping device and a Bourdon tube both in a tee configuration) was placed between the pump outlet and the injector to ensure a pulseless delivery of the chromatographic eluate to the flow-sensitive electrochemical detectors.

A PAR Model 310 detector (EG&G, Princeton Applied Research, Princeton, NJ. U.S.A.) was used as polarographic detector. The previously described [10] glassy carbon wall jet electrode was used as voltammetric detector. Both detectors, operating in the amperometric mode, were controlled by a PAR 174 A polarographic analyser.

Materials

Solvents used were HPLC grade (Carlo Erba). Allopurinol, oxipurinol and uric acid were obtained from Sigma. All the other chemicals were analytical reagent grade.

The phosphate buffer (pH 6.1, 0.025 *M*) used in the mobile phase was filtered through a 0.45 μm membrane before use.

Chromatographic conditions

The mobile phase was a 0.025 *M* phosphate buffer pH 6.1 with 6—8% of methanol added unless otherwise specified. It was degassed, before use, by conventional methods.

The flow-rate was normally 1.5 ml/min and the injection volume 20 μl. All separations were run at room temperature. Quantitations were done by an external standard method. This choice was conditioned by the fact that it was not easy to find a compound with the same polarographic behaviour as the studied drugs that could act as internal standard.

Sample preparation

A sample clean-up procedure similar to that described in ref. 11 for seven model drugs has been found to work satisfactorily in the present case.

Urine. A 2-ml volume of urine was mixed with 0.1 ml of 15% (w/w) zinc sulphate, 0.4 ml of saturated barium hydroxide and 2 ml of methanol. After each reagent addition the sample was vortex-mixed for a few minutes and was finally centrifuged at about 1200 *g* for 5 min. The supernatant was diluted, as necessary, with the mobile phase and injected. (Unless otherwise specified the final dilution ratios were 1:40 for voltammetric detection and 1:20 for polarographic detection.) Alternatively, urine could be injected directly into the chromatographic system after dilution with the mobile phase. In this case, however, the use of a precolumn is recommended to prolong the life of the analytical column.

Serum (or plasma). To 1 ml of serum the following reagents were added in sequence: 0.1 ml of 15% (w/w) zinc sulphate, 0.2 ml of saturated barium hydroxide, 0.1 ml of 0.5 mol/l phosphate buffer pH 6.1, 1 ml of methanol. After each addition the sample was briefly vortex-mixed and finally centrifuged at approximately 1200 *g* for 5 min. The supernatant was ready for direct chromatographic analysis. Alternatively (e.g. when a preconcentration of the sample was required) the following procedure was adopted: 0.5 ml of serum was extracted twice with 5 ml of diethyl ether—isopropanol (6:1, v/v) mixture. The combined extracts were concentrated to dryness, reconstituted with 50 μl of mobile phase and 20 μl were injected directly.

RESULTS AND DISCUSSION

HPLC with electrochemical detection

Allopurinol, oxipurinol and uric acid give rise [10] to anodic waves at mercury electrodes due to a depolarization effect on the mercury oxidation caused by the formation of sparingly soluble compounds with mercury ions. This peculiarity can permit the use of an HPLC polarographic detector operat-

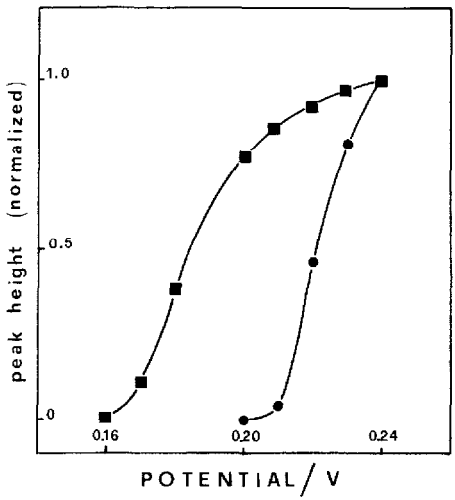

Fig. 1. Current—potential profiles (hydrodynamic conditions) for allopurinol (160 ng) and oxipurinol (220 ng). Injected volume, 20 μl; column, C_8 10 μm (250 × 4.6 mm I.D.); mobile phase, phosphate buffer—methanol (92:8); drop time, 1 sec; flow rate, 1.5 ml/min; detection mode, sampled d.c.

ing in the oxidative mode. In this way the unique advantage of a polarographic detector, i.e. the continuously renewed electrode surface, can be coupled to the main advantage of the oxidative operating mode, i.e. no requirement for the deoxygenation of the mobile phase (the main deterrent in using an electrochemical detector in the reductive mode).

The current—potential profile for allopurinol and oxipurinol obtained at a dropping mercury electrode detector in hydrodynamic conditions is shown in Fig. 1. An applied potential higher than +0.245 V caused an abnormal increase of the noise, which lead to a decrease of the signal-to-noise (S/N) ratio; the peak currents were then normalized to the value obtained at +0.24 V which represents the optimum value for the applied potential. From Fig. 1 it can be seen that, if desired, oxipurinol may be selectively determined by setting the potential at +0.20 V with only a 20% decrease in sensitivity. This kind of selectivity can be rarely obtained with other detectors such as the UV detector.

Detection limits calculated at S/N = 2 (in the following experimental conditions: flow-rate 1.5 ml/min; drop time 1 sec; drop size setting at the PAR 310 "small"; applied potential +0.24 V vs. Ag/AgCl) are around 0.8 ng. This value can be lowered by a factor of approximately 2 by using a mercury drop of higher area (drop size setting "medium").

Calibration plots, generated in the range 2—2000 ng, were found to be linear with regression coefficients higher than 0.999 for both compounds.

Allopurinol and oxipurinol were both found [10] to be electroactive on the glassy carbon electrode and a mechanistic pathway for their oxidation has been suggested. At the same time it is known that uric acid can be easily oxidized on glassy carbon and can be determined by HPLC with electrochemical detection [6].

The analytical applications of an HPLC method with voltammetric and/or polarographic detection to the analysis of real matrices are described below.

Analysis of urine and serum samples

The procedure described in the experimental section for the deproteinization of urine and serum has been found effective, simple and inexpensive. The resultant supernatant is free of visible residues and both types of sample can be analyzed without a guard column. No change in the performance or in the working pressure of the column has been observed in the course of the present work.

The recovery of the drugs under examination increases on increasing the quantity of methanol added to the sample until a plateau is reached. Fig. 2 illustrates the pattern trend in urine. In this case recoveries were determined on urine containing added allopurinol and oxipurinol, by comparison of the chromatograms obtained on treated and untreated urine aliquots both at the same final dilution ratio (1:20) obtained by adding mobile phase. The recovery from serum was estimated on spiked samples by direct comparison with drug standards. Blank (control) samples of serum and urine taken from different subjects showed no interference from endogenous compounds for either allopurinol or oxipurinol. Other drugs such as probenecide and phenylbutazone, which gouty patients could take concurrently with allpurinol, cannot interfere with the present assay because of their electrochemical inactivity. Theobromine and theophylline which are voltammetrically, but not polarographically, electroactive were also found not to interfere.

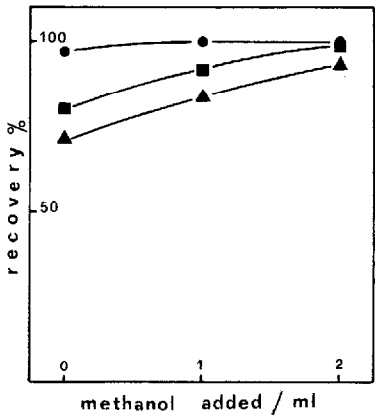

Fig. 2. Recovery of the studied compounds from urine as a function of the quantity of methanol added. The volume of treated urine is 2 ml; pretreatment was as described in the experimental section. (■), oxipurinol; (●), allopurinol; (▲), uric acid.

Figs. 3 and 4 show typical chromatograms of urine samples, taken from a patient under treatment with 300 mg/day of allopurinol, recorded using a voltammetric detector and a polarographic detector, respectively. Under the given experimental conditions the polarographic detector appears more selective in particular for the first part of the chromatogram where the uric acid peak (retention time ca. 2.8 min) is eluted. Moreover, in the case of polarographic detection, no major peak appears after the elution of allopurinol while in the case of voltammetric detection the last eluting peak occurs at about 19.5 min.

Improved resolution of the uric acid peak (in the case of voltammetric

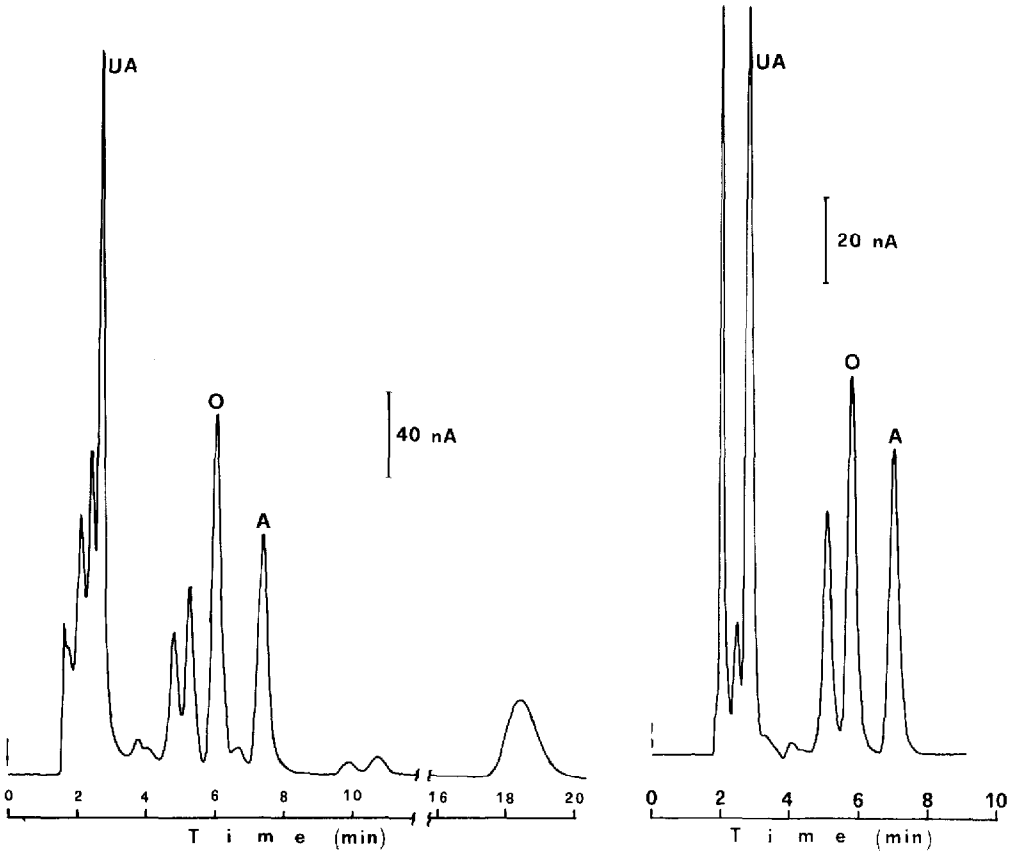

Fig. 3. Chromatogram of a urine sample from a patient under treatment with 300 mg/day of allopurinol (the final dilution ratio of urine was 1:40). Glassy carbon wall jet detector operating at +1.2 V vs. SCE in the d.c. mode. Mobile phase, phosphate buffer—methanol (94:6); flow-rate, 1.5 ml/min. UA = uric acid; O = oxipurinol; A = allopurinol.

Fig. 4. Chromatogram of a urine sample from a patient under treatment with 300 mg/day of allopurinol obtained by a polarographic detector operating in the oxidative mode (the final dilution ratio of urine was 1:20). Experimental conditions: applied potential, +0.24 V vs. Ag/AgCl; other conditions as in Fig. 1. Peak notation as in Fig. 3.

detection) can be achieved simply by changing the applied potential. Profile A in Fig. 5, for example, shows how the chromatogram of Fig. 3 changes when the applied potential is reduced from +1.2 V to +0.7 V vs. standard calomel electrode (SCE). The uric acid peak is now completely resolved but, at the same time, any signal of allopurinol and oxipurinol is completely lost. Intermediate potential values gave a different degree of selectivity: for example, at +1.0 V uric acid and oxipurinol were determined simultaneously. Of course the simultaneous determination of allopurinol, oxipurinol and uric acid in urine by HPLC—voltammetric detection can be obtained by the most suitable choice of the mobile phase (e.g. using an ion-pair agent) which in the present case has been optimized for polarographic detection. Alternatively, a dual-electrode (parallel configuration) cell could be used in which the first electrode is held a +0.7 V (uric acid) and the second at +1.2 V (allopurinol and oxipurinol).

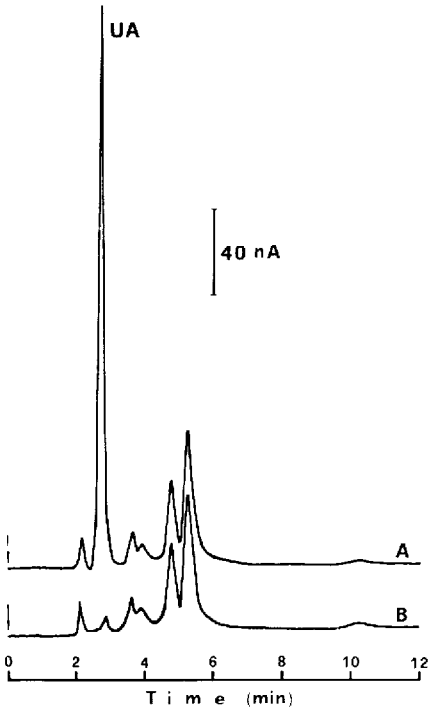

Fig. 5. (A) Chromatogram obtained in the same conditions as Fig. 4 except for the applied potential which, in the present case, is +0.7 V vs. SCE. (B) Chromatogram obtained in the same conditions as chromatogram A on a urine sample treated, before the deproteinization step, with the enzyme uricase.

Since uric acid is an endogenous component of human body fluids it was necessary to ascertain the purity of the relevant chromatographic peak (for both detection methods); this was simply done by treating the sample, before the deproteinization step, with the enzyme uricase. After this treatment a profile such as B in Fig. 5 was obtained which shows a nearly flat baseline at the retention time of uric acid. In addition, the urinary, or serum (see later), uric acid concentrations obtained with the HPLC method were found to be in agreement (within ± 8%) with those obtained by a conventional enzymatic method [12]. Figs. 6 and 7 show typical chromatograms relevant to serum samples containing allopurinol and oxipurinol recorded with a polarographic and voltammetric detector, respectively. Again the polarographic detector appears more specific even if, in this case, the uric acid peak appears well resolved also on the voltammetric detector response.

To evaluate precision known amounts (2—10 μg/ml) of allopurinol and oxipurinol were added to urine and serum samples which were then treated as described in the experimental section. The coefficient of variation observed within-day for both compounds in serum and urine ranged from 3% to 4%; the day-to-day precision was in the range 3.5—5%.

Detection limits for allopurinol and oxipurinol with both methods of detection and for both matrices were found to be practically the same: ca. 0.2 μg/ml. This value is well below the usual serum [2] or urinary concentrations of the studied drugs associated with the usual therapeutic doses.

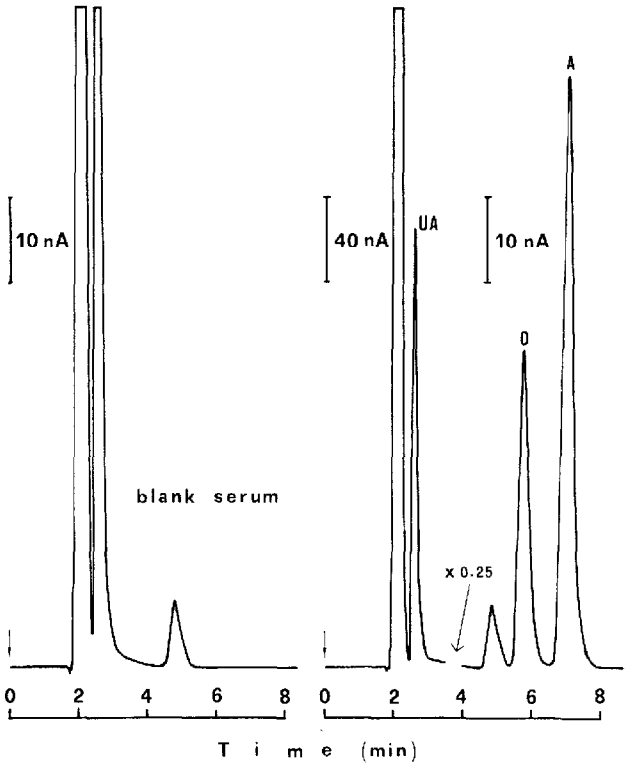

Fig. 6. Chromatograms relevant to a blank serum sample (left) and to a serum sample with added oxipurinol and allopurinol (right). Polarographic detector. Experimental conditions as in Fig. 5. Peak notation as in Fig. 3. The arrow at about 3.5 min indicates a sensitivity change. Injected quantities: allopurinol 120 ng; oxipurinol 80 ng.

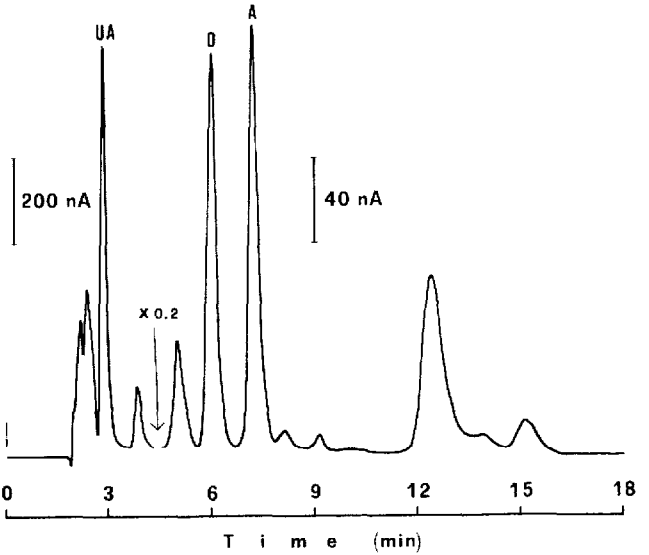

Fig. 7. Chromatogram of a serum sample with added oxipurinol and allopurinol. Voltammetric detector. Experimental conditions and peak notation as in Fig. 3. The arrow at about 4 min indicates a sensitivity change. Injected quantities: allopurinol 130 ng; oxipurinol 138 ng.

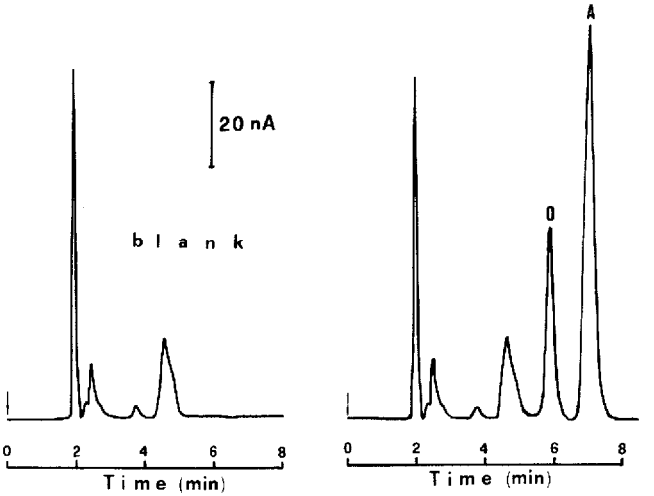

Fig. 8. Chromatograms relevant to serum extracts. Polarographic detector. Left: chromatogram relevant to the extract from a blank serum sample. Right: chromatogram relevant to the extract from a serum sample containing 0.45 μg/ml of oxipurinol and 0.7 μg/ml allopurinol. Experimental conditions and peak notation as in Fig. 6.

However, if desired, the previously reported detection limits can be lowered by about one order of magnitude (i.e. to about 10—20 ng/ml) by means of the extraction procedure described in the experimental section. This procedure has been evaluated only for serum because it presents a lower concentration of the drugs (with respect to urine) and is more important from a clinical point of view. Fig. 8 shows typical chromatograms (polarographic detector) relevant to an extract from a serum blank sample and from a serum sample containing 0.45 μg/ml of oxipurinol and 0.7 μg/ml of allopurinol. The recovery of the proposed procedure (estimated at the above concentration levels by direct comparison to standards that were not extracted) was found to be quite satisfactory, for both compounds being 95 ± 4%.

CONCLUSION

The results presented in this paper clearly indicate that a polarographic detector can be successfully employed in the oxidative mode for those compounds which form insoluble salts with mercury. The detector appears even more specific than a voltammetric detector and possesses the unique peculiarity of a continuously renewable electrode surface. The only disadvantage is perhaps represented by the necessity of handling mercury which could discourage some potential users. It is interesting to note that an anodic behaviour similar to that of allopurinol, oxipurinol and uric acid is presented [13] by a considerably large number of compounds of biological or pharmaceutical interest such as most purines and pyrimidines, thiocompounds (like cysteine, cystine, glutathione, thiobarbiturates, etc.), sulphadrugs, porphyrins and so on. A possible renewed interest could be given to polarographic detectors (whose main use has been, up to now, for reducible compounds) since the detection mode presented here can extend considerably the utilization of mercury in the anodic range.

In this laboratory a study is in progress [14] dealing with the detection of antineoplastic agents such as 5-fluoro- and 5-bromouracil, methotrexate, 5-mercaptopurine and 2-thioguanine.

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