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DETERMINATION OF PLASMA AND URINE LEVELS OF A NEW ANTI-INFLAMMATORY AGENT, 4,5-BIS-(4-METHOXYPHENYL)-2-(2-HYDROXYMETHYLSULFINYL)-IMIDAZOLE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL OR ULTRAVIOLET DETECTION

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SUMMARY

The determination of a new anti-inflammatory substance, 4,5-bis-(4-methoxyphenyl)-2-(2-hydroxyethylsulfinyl)-imidazole, in plasma and urine by high-performance liquid chromatography is described. Ultraviolet and electrochemical detection modes are compared and special consideration is given to the mechanism of the electrochemical reaction. The site of oxidation in the molecule seems to be an aliphatic hydroxyl group yielding a carboxyl function in a four-electron transfer reaction. Plasma levels and urinary excretion after an oral dose of 250 mg to two male volunteers have been measured.

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ECD) continues to gain in popularity for the sensitive and selective determination of trace components in complex biological samples. The sensitivity of this method of detection in the oxidative mode has been demonstrated by Kissinger et al. [1], who designed and built a system with a carbon-paste electrode, capable of measuring picograms of catecholamines. Other groups have constructed cells by using glassy carbon electrodes thus making this mode of detection suitable for non-aqueous eluents [2—4]. The selectivity of ECD is achieved by detecting only those compounds which are electrochemically active at the operating potential chosen. This is of particular interest in the analysis of biological samples. Many of the substances usually interfering which are co-extracted in the work-up of plasma or urine samples are electrochemically inactive and will therefore not interfere in ECD. Thus the suit-

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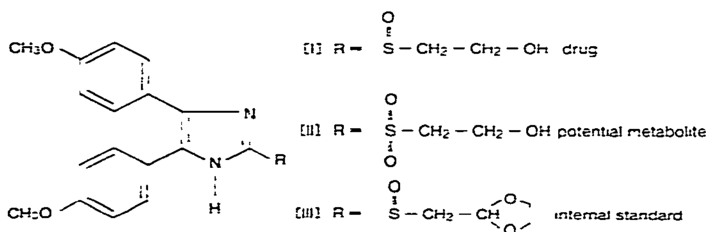
ability of the electrochemical detector to a given problem ultimately depends on the voltammetric characteristics of the compound to be detected in a suitable mobile phase and at a suitable electrode potential.

Since the electrooxidative determination of catecholamines many more classes of compounds have been investigated and found suitable for electrochemical detection, for example, tryptophan metabolites [5], alkaloids [6], β-blockers and their metabolites [7, 8], aromatic amine carcinogens [9], and ascorbic acid [10, 11].

The present report describes a sensitive and specific method of determination for a new substance with anti-inflammatory activity: 4,5-bis-(4-methoxyphenyl)-2-(2-hydroxyethylsulfinyl)-imidazole (compound I). ECD is compared to ultraviolet detection of the drug in plasma and urine extracts. Special interest was focussed on investigating the site of the electrochemical reaction in the molecule.



EXPERIMENTAL

Subjects and medication

Two healthy male volunteers (36 and 26 years of age and 65 and 96 kg body weight, respectively) were both given 250 mg of (I) orally in capsule form in the morning on an empty stomach. Breakfast was not allowed until 2 h after administration. Blood samples were taken at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24 and 48 h after the drug administration. The samples were immediately centrifuged and the plasma stored frozen until analysis. Urine was quantitatively collected at 2, 4, 6, 8, 10, 12, 24 and 48 h after administration and kept frozen until analysis.

Chemicals

Methanol, diethyl ether and disodium hydrogen phosphate were all of analytical-reagent grade (Merck, Darmstadt, G.F.R.) and used without further purification.

Compound (I), the internal standard (III) and the possible metabolite (II) of (I) were synthesized by Dr. Niedballa (Schering) and stored dissolved in methanol in concentrations of 100, 10 and 1 μg/ml, respectively.

Glassware

All glassware used in the extraction procedure was cleaned with chromic acid and washed with distilled water before use.

Extraction procedure

One millilitre of plasma or urine was pipetted into an 8-ml stoppered test-tube and 0.5 μg of internal standard and 3 ml of diethyl ether were added. After thorough mixing on a Vortex mixer for 1 min and centrifugation at 1200 *g* for 5 min, the organic phase was transferred to another test-tube and evaporated to dryness. The residue was dissolved in 200 μl of the HPLC mobile phase. After centrifugation (5 min, 1200 *g*) — only in the case of plasma extracts — 150 μl were injected for analysis.

The extraction efficiency was determined with 1-ml plasma and urine samples containing 0.5 μg of (I) or of internal standard (III) (n = 5). Peak heights were measured and correlated with peak heights of pure substances.

Chromatographic system

The HPLC system consisted of a solvent delivery pump (Waters, Königstein, G.F.R., type 6000A), a LiChrosorb RP-18 chromatographic column (10 μm particle size, 250 × 4.6 mm) with precolumn (40 × 4.6 mm; Knauer, Berlin, G.F.R.) and an electrochemical detector (E 611, cell 1096/2; Metrohm, Filderstadt, G.F.R.) using a glassy carbon working electrode and an Ag/AgCl/KCl reference electrode. Alternatively, a UV detector with variable wavelength (Schoeffel SF 770) was used. Injection was accomplished with a Rheodyne RH 7120 system or an automatic injector (WISP, Waters). The mobile phase consisted of methanol—water (1:1, v/v) with 0.01 *M* disodium hydrogen phosphate per litre. The eluent was degassed under reduced pressure before use. The chromatographic system was operated at ambient temperature with an eluent flow-rate of 2 ml/min.

The electrochemical potential of the working electrode was set at +1.0 V against the reference electrode. The current range used was 75—200 μA according to the concentration of the drug. The UV detector was set at 280 nm, the absorption maximum of (I). The detector signals were converted to chromatographic traces by a W + W recorder (Basle, Switzerland) at an input voltage of 1000 and 100 mV, respectively.

Calibration curve

Standard curves were constructed with 1-ml blank plasma and urine samples containing 0, 0.05, 0.1, 0.5, 0.75, 1, 1.5, 2, 2.5 and 5 μg of (I) and 0.5 μg of internal standard. These samples were extracted by the method described above. Peak heights of internal standard and drug were measured and the calibration curve [peak height ratio of (I):internal standard (III) versus the concentration of (I)] was constructed.

Unknown plasma and urine samples were processed together with five calibration points [5.0, 2.5, 1.0, 0.5 and 0.1 μg of (I)] which were used to correct for inter-assay variability.

The overall accuracy of the assay was calculated from five consecutive determinations of 0.5 μg of (I) in plasma and urine.

Electrochemical reaction

To specify the site of electrochemical reaction in the molecule, response (peak height) was measured as a function of the potential of the working

electrode for (I) and for two model substances in which only one structural element had been altered (see structural formulae). By this procedure contributions from background current and other residual currents which arise in scanning voltammetry are conveniently avoided [6].

RESULTS AND DISCUSSION

A highly sensitive and selective method for the determination of the new anti-inflammatory substance (I) in plasma and urine is described utilizing HPLC with electrochemical (plasma samples) or UV (urine samples) detection. Extraction from biological specimens is performed with diethyl ether. The recovery of this procedure was found to be about 40% for (I) and for the internal standard (III), in both plasma and urine (see Table I).

TABLE I

EXTRACTION RECOVERIES OF (I) AND INTERNAL STANDARD (III)

Recoveries were determined by extracting 1 ml of plasma or urine spiked with 0.5 μg of (I) or of internal standard, and comparing the ECD peak heights to those of non-extracted material.

Sample	Recovery (%)	
	(I)	Internal standard (III)
Plasma	41.2	40.8
	43.2	45.0
	38.4	41.6
	42.6	44.2
	40.4	42.2
Mean ± S.D.	41.2 ± 1.9	42.8 ± 1.8
Urine	33.6	46.0
	28.4	24.8
	41.2	40.0
	38.2	40.4
	41.8	45.6
Mean ± S.D.	36.6 ± 5.6	39.4 ± 8.6

Matrix constituents and possible co-extracted metabolites of (I) are then separated from the drug by HPLC using a reversed-phase system (see Fig. 1). For detection two types of systems were compared, electrochemical and UV detectors. As shown in Figs. 1—4, ECD is suitable for plasma and urine samples whereas UV detection can only be used for urine and not for plasma samples. The reason for this is the UV-absorbing plasma constituents which interfere in the optical mode but which are not oxidized and detected at the applied potential in the electrochemical mode.

Concentrations of (I) in plasma and urine were determined by comparing the peak heights of the drug and the internal standard added before extraction. Typical calibration curves of the assay for ECD and UV detection are described by the equations given in Table II.

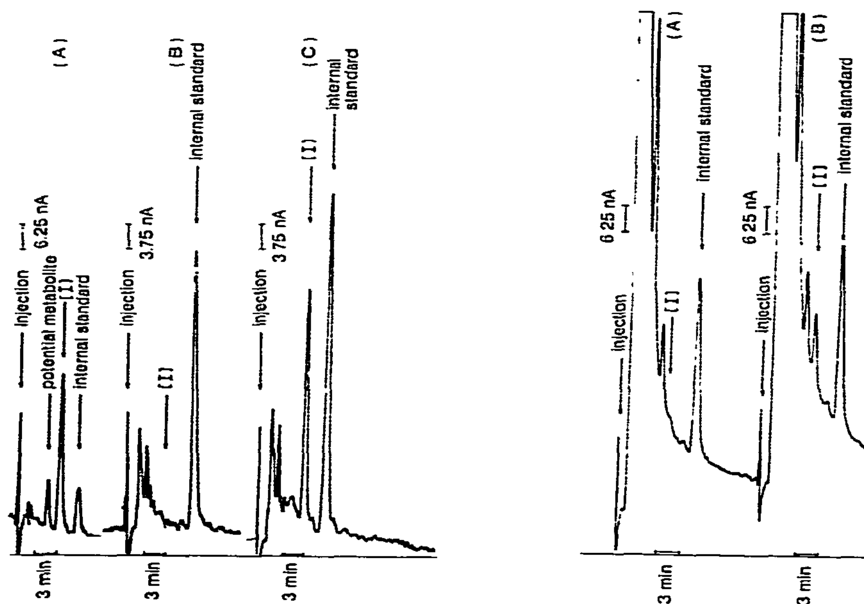


Fig. 1. HPLC chromatograms (electrochemical detector) of (A) 50 ng each of (I), its potential metabolite (II) and the internal standard (III), and of blank plasma samples spiked with (B) 500 ng of the internal standard and (C) 100 ng of (I) and 500 ng of the internal standard.

Fig. 2 HPLC chromatograms (electrochemical detector) of blank urine samples spiked with (A) 500 ng of internal standard, and (B) 50 ng of (I) and 500 ng of internal standard.

TABLE II

MATHEMATICAL EQUATIONS OF THE CALIBRATION CURVES FOR THE DETERMINATION OF UNKNOWN (I) CONCENTRATIONS

The curves were obtained by spiking 1 ml of plasma or urine with 0.5 μg of internal standard and various amounts of (I).

Specimen	Detection	Calibration curve*	Correlation coefficient
Plasma	EC	$y = 0.32 + 5.77x$**	0.9993
	UV	Not determined	
Urine	EC	$y = 0.16 + 6.48x$***	0.9993
	UV	$y = 0.05 + 2.69x$***	0.9992

*y = (peak height of (I))/(peak height of internal standard); x = concentration of (I).
**n = 11.
***n = 10.

The overall accuracy of the assay expressed as standard deviation of five consecutive determinations of 0.5 μg of (I) is shown in Table III. As can be seen from this table, UV detection is more suitable for urine samples than ECD because of better reproducibility.

The detection limit after extraction of 1 ml of plasma or urine is below

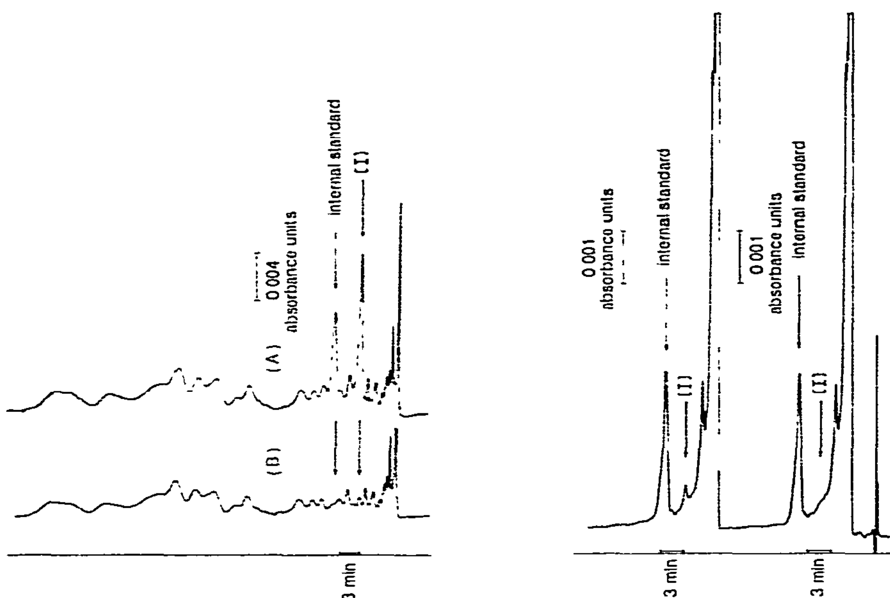


Fig. 3. HPLC chromatograms (UV detector) of (A) blank plasma sample spiked with 0.5 μg each of (I) and internal standard, and (B) blank plasma sample.

Fig. 4. HPLC chromatograms (UV detector) of blank urine samples spiked with (left) 0.5 μg of internal standard and 50 ng of (I) and (right) 0.5 μg of internal standard.

TABLE III

PRECISION OF THE ASSAY

Precision was calculated from five consecutive determinations of 0.5 μg of (I) in 1 ml of plasma or urine.

Specimen	Coefficient of variation	
	UV detection	EC detection
Plasma	Not determined	3.0
Urine	2.1	15.0

10 ng/ml for ECD and about 10 ng/ml for the UV mode. The limit may possibly be increased further by extracting larger sample volumes, by increasing the extraction efficiency (90% when extracting samples three times) and by lowering the applied current to about 5 nA.

Study of plasma and urine levels

(I) reached its maximum plasma level of 713 ± 163 ng/ml in the two test subjects 4 h after administration (Fig. 5). At 12, 24 and 48 h after dosing the concentration had fallen to 273 ± 47, 208 ± 32 and 36 ± 6 ng/ml, respectively. Renal excretion of unchanged drug amounted to 1.1 ± 0.2 mg in the first two days, which is equal to 0.4% of the dose (Fig. 6). Detailed pharmacokinetics of this drug including dose dependency will be reported elsewhere [12].

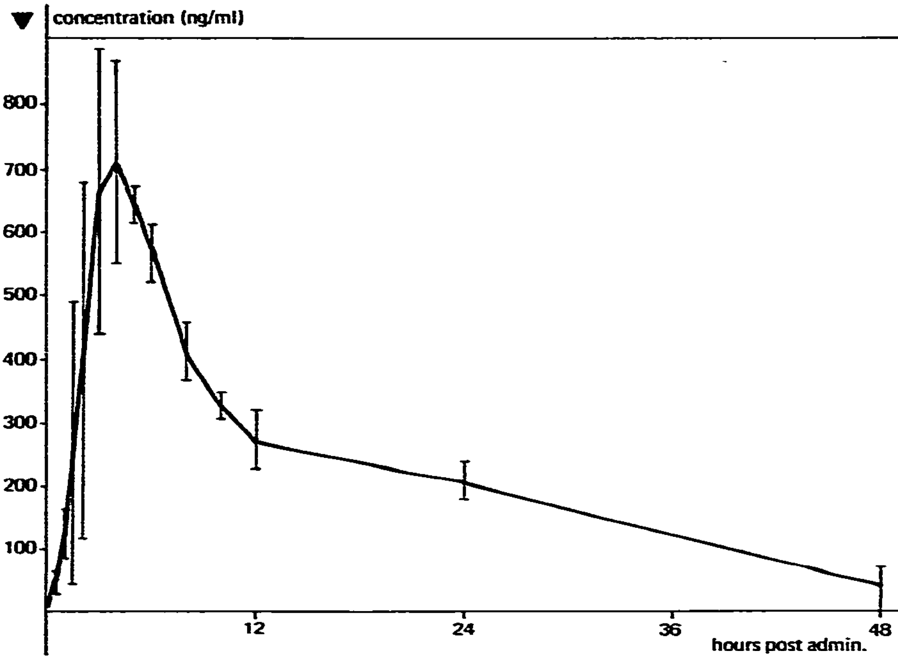


Fig. 5. Plasma level of (I) (mean ± S.D.) after oral administration of 250 mg to two healthy male volunteers.

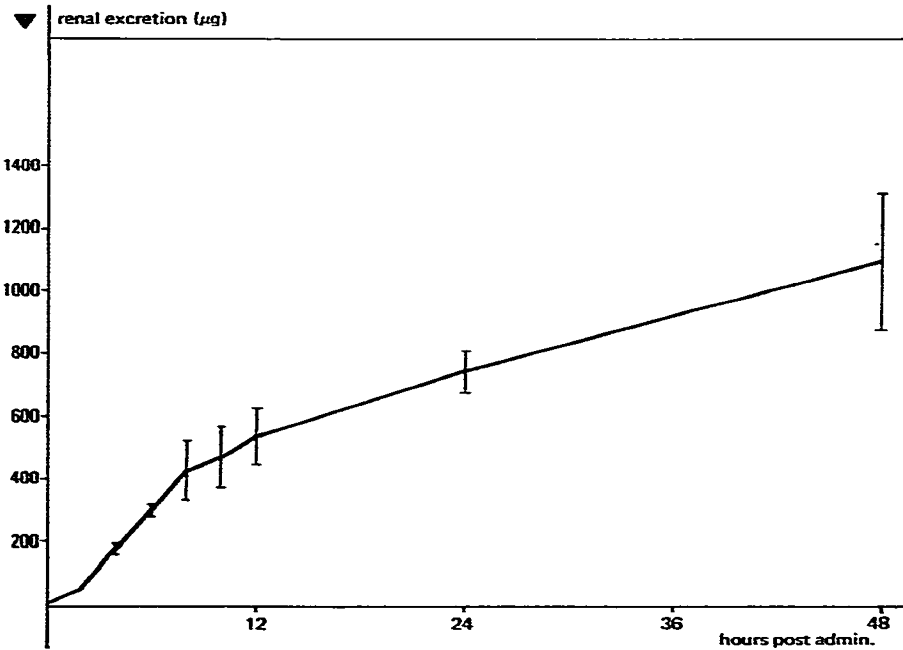


Fig. 6. Urinary excretion of (I) (mean ± S.D.) after oral administration of 250 mg to two healthy male volunteers.

Electrochemical reaction

At an electrode potential of +1.0 V used in the ECD mode substances eluted from the chromatographic column are changed by oxidation. (I) is prone to oxidation at different sites of the molecule. First, the primary alcohol group of the side-chain may be oxidized to the aldehyde or the carboxylic acid [13] and, second, the sulfinyl group may be converted to sulfonyl [14, 15], whereas the imidazole ring is quite stable to oxidation [16].

To clarify the site of oxidation two model compounds have been examined which differ from (I) only in one part of the molecule (see structural formulae). Current—potential curves of these three substances have been obtained by plotting peak heights of 0.5 μg of each vs. the potential applied (Fig. 7). From these curves the following conclusions can be drawn: (1) Compound (I) is more easily oxidized than (II). (2) (III) is more easily oxidized than (II), but less than (I). (3) In (I) and (II) the same amounts of electrons are exchanged (same peak heights in the end stage of oxidation). (4) In (III) only half of the electrons are transferred compared to (I) and (II).

If the sulfinyl group were oxidized, the peak heights of (I) and (III) would have to be the same and (II) should give no signal at all. With the primary alcohol group being the reaction centre, (I) and (II) have to yield the same response in the final stage of oxidation. If the reaction were to produce the aldehyde, (III) should give no signal, because this compound is already an aldehyde (in the form of its acetal). Therefore, the oxidation has to proceed from the alcohol via the aldehyde to the carboxylic acid. So in (I) and (II) there are four electrons transferred whereas in (III) there are only two.

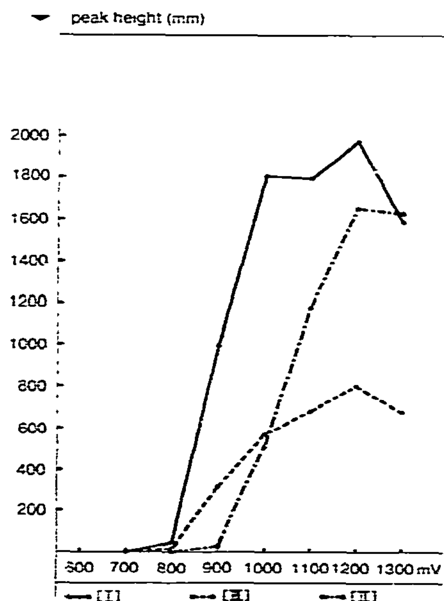
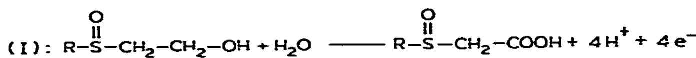
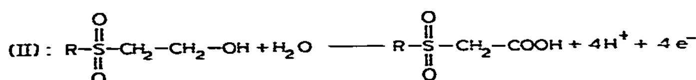
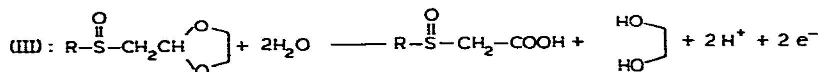


Fig. 7. Electrochemical response (peak height) of 0.5 μg each of drug (I), its potential metabolite (II), and internal standard (III) at various potential settings.







As (I) is more easily oxidized than (II), the reaction has to proceed under the influence of the sulfinyl group, possibly also under the mediation of the imidazole ring. One could think of a cyclic intermediate with the electron exchange starting from the $\underset{\underset{O}{\|}}{-S-}$ group.

In the literature little is known about the mechanism of electrochemical detection of most of the chemical classes studied. Catecholamines and substituted derivatives have been shown to yield benzoquinones with ring closure of the amine-containing side-chain [17—19]. Tryptophan and its metabolites seem to be oxidized at 5-hydroxy groups on the phenol ring or at higher voltages applied at the ring nitrogen atom to give N^+ [20]. Morphine is reported possibly to dimerize after a one-electron transfer to yield pseudomorphine, which is further oxidized in a second step [21]. The oxidation of aliphatic hydroxyl groups as described in the present report seems to be a new way of reaction in electrochemical detection.

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