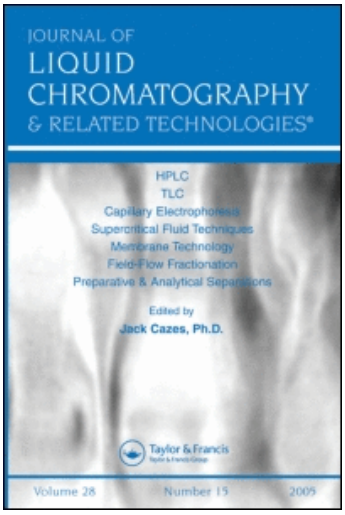

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The Determination of a New Trifluorinated Quinolone, Fleroxacin, Its N-Demethyl, and N-Oxide Metabolites in Plasma and Urine by High Performance Liquid Chromatography with Fluorescence Detection

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THE DETERMINATION OF A NEW TRIFLUORINATED QUINOLONE, FLEROXACIN, ITS N-DEMETHYL, AND N-OXIDE METABOLITES IN PLASMA AND URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A liquid chromatographic method is described for the determination of the new fluoroquinolone Ro 23-6240 and its N-demethyl and N-oxide metabolites in plasma and urine. The three substances were extracted from aqueous solution with dichloromethane/isopropanol containing sodium dodecyl sulphate. After evaporation and reconstitution, samples were analysed on a reversed-phase column using ion pair chromatography and fluorescence detection. The limit of quantification was 10-20 ng/ml (RSD 4 %) using a 0.5 ml plasma sample, and the inter assay precision was 3-10 % over the concentration range 50 ng/ml to 20 µg/ml. Recovery from plasma was 81 % (RSD 10 %) over the range 10 ng/ml to 5 µg/ml. The method has been applied successfully to the analysis of several thousand samples from human pharmacokinetic studies. Care has to be taken to avoid exposure of samples to direct sunlight, and the use of opaque vessels for sample storage and handling is recommended.

INTRODUCTION

The quinolone acid derivative Fleroxacin (I; 6,8-difluoro-1-(2-fluoroethyl)-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinoline-carboxylic acid, Fig. 1) is a potent and broad spectrum antibacterial against both gram-positive and gram-negative bacteria [1]. In order to obtain the pharmacokinetic parameters of this substance in man, an analytical method was required for the determination of I and its major metabolites, the N-oxide and N-demethyl analogues (II and III), in plasma and urine. An HPLC method has already been described for this substance, but this was restricted in application to animal kinetics and metabolism, and the lowest validated concentration reported (500 ng/ml) is insufficient for human pharmacokinetics [2].

The method described here for the parent drug in plasma, with a quantification limit of 20 ng/ml, is based on extraction at neutral pH followed by ion pair HPLC on a reversed phase column with fluorescence detection. For urine analysis, in order to extract the metabolites efficiently, an ion pair reagent, sodium dodecyl sulphate, had to be incorporated in the extraction solvent.

Similar methods have already been reported for the quinoline carboxylic acids nalidixic acid [3], and ciprofloxacin [4,5,6,7,8]. A recently published method for pipemidic acid [9] uses pre-column methylation followed by normal phase chromatography with perchloric acid in the methanol/chloroform mobile phase.

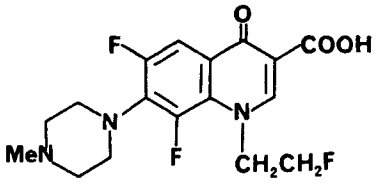
EXPERIMENTAL

Reagents and Materials

Methanol, potassium dihydrogen phosphate, disodium hydrogen phosphate were p.a. grade from Fluka AG (Buchs, Switzerland); isopropyl alcohol, dichloromethane, acetic acid, and sodium hydroxide were puriss. p.a. grade, also from Fluka; tetrabutylammonium hydrogensulphate and sodium dodecyl (lauryl) sulphate were puriss. grade, also from Fluka; hydrochloric acid (0.1 M Titrisol) was from Merck (Darmstadt, FRG); technical grade isopropyl acetate was distilled and the 86°-88°C fraction used. Compounds I, II, III, and IV were synthesized by Kyorin Pharmaceutical Co. Ltd., Tochigi, Japan.

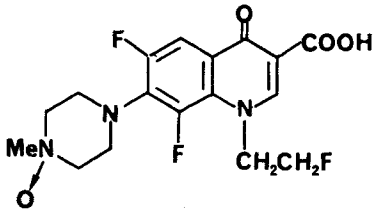
Ro 23-6240
AM-833

I



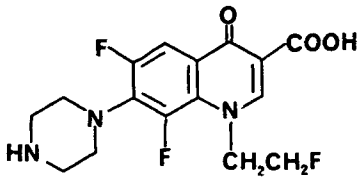
Ro 19-7728

II



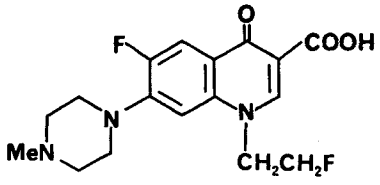
Ro 19-7513

III



AM-735

IV



Pipemidic acid

V

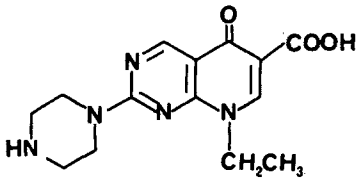


FIGURE 1 Structures of compounds

Preparation of Standard Solutions

Compound I was dissolved in sodium hydroxide solution (0.002 M) using ultrasonication (200 μg/ml). This stock solution was diluted with sodium hydroxide solution (0.002 M) to give a series of standards in the concentration range 1 μg/ml to 200 μg/ml. Each of these standards (100 μl aliquots) was mixed with control plasma (9.9 ml) to provide plasma standards in the concentration range 10 ng/ml - 2000 ng/ml.

For the preparation of urine standards, a stock solution containing 1000 μg/ml of I and 200 μg/ml of both II and III in 0.002 M sodium hydroxide was made up. From this solution, by dilution with control urine, urine standards were prepared containing I, II, and III within the concentration ranges 200 μg/ml to 1 μg/ml for I, and 40 μg/ml to 0.2 μg/ml for II and III.

All solutions were prepared in brown glass vessels. Plasma and urine standards were stored deep-frozen (-20°C) until required, and the sodium hydroxide solutions were kept at 0-4°C and renewed at fortnightly intervals.

Sample Preparation Procedures

Plasma (method A) : Frozen samples were allowed to thaw at room temperature and then homogenized on a vortex mixer. Two aliquots (0.5 ml) were taken; one was refrozen for analysis on the following day and the other was immediately processed as follows. Internal standard solution (IV, 1000 ng/ml, 0.01 M HCl, 100 μl) [or, for the pre-dose sample, 100 μl twice distilled water instead of internal standard], phosphate buffer (Sörensen, pH 7.5, 1.0 ml), and dichloromethane/isopropanol (7/3, v/v, 7.0 ml) were added to the plasma aliquot. After head-over-head extraction (70 rotations/minute for 10 minutes), and centrifugation (2000 g, 10 minutes), the aqueous phase was removed and an aliquot (5 ml) of the organic phase was transferred into a conical brown glass tube. The organic phase was evaporated to dryness (under a stream of nitrogen or under water pump vacuum at 35°C) and reconstituted in mobile phase (in 500 μl with vigorous mixing [vortex]; see below).

Urine (method B) : Frozen samples were thawed, aliquots taken as described above, and the samples processed as follows. Pipemidic acid (V), internal standard solution (1 mg/ml, 0.01 M HCl, 100 µl) [for pre-dose sample, 100 µl water], acetic acid (1 M, 0.5 ml), sodium dodecyl sulphate (25 mM in water, 0.5 ml), and dichloromethane/isopropyl alcohol (7/3 v/v, 7.0 ml) were added to the urine aliquots. The procedure for extraction, centrifugation, and evaporation was as described for plasma. The evaporated extracts were dissolved in mobile phase, the volumes ranging from 0.5 ml to 5 ml, depending upon the concentrations expected.

Calibration and Quality Control

On each analysis day, 6 to 8 of the plasma or urine standards (see above) covering the expected concentration range were thawed (0.5 ml aliquots) and extracted as described above. At the same time, 2-3 independently prepared QC samples, covering the calibration range, were extracted.

Chromatographic Procedures

The HPLC system consisted of the following components : Model 410 pump, SFM-23 fluorescence detector (Kontron, Zürich, Switzerland); automatic injector (Waters WISP, Brechbühler, Schlieren, Switzerland, or Kontron MSI-600T, Kontron); Spectroflow 773 UV detector (Kratos, Basel, Switzerland); SP 4200 computing integrator and 4100 D minifile (Spectra Physics, Basel, Switzerland); HPLC column block heater (Ercatech, model 7930); HPLC column 250 x 4.6 mm stainless steel tube filled with Toyo Soda TSK-Gel ODS 120T (5 µ), or 250 x 4.0 mm packed by ourselves with the same material (LKB, Littau, Switzerland). The mobile phases were :

(i) For plasma : tetrabutylammonium hydrogensulphate (5 mM, 1.72 g dissolved in 1000 ml bidistilled water) 79 %, methanol 21 % (v/v); column at 27°C, flow rate 0.8 ml/min.

(ii) For urine : as for plasma, except volume proportions 74.5 % and 25.5 %, respectively; column at room temperature, flow rate 0.8 ml/min.

The fluorescence detector was operated at excitation and emission wavelengths of 290 nm and 450 nm, respectively, and the UV detector at 290 nm.

With this procedure it was possible to analyse about 48 samples in 24 hours.

Calculations

Peak height ratios of the calibration samples are plotted against concentration, and a weighted quadratic regression is computed $\left(\text{weighting factor} : \frac{1}{\left(\text{peak height ratio}\right)^2}\right)$

Concentrations of drug and metabolites in unknown samples are obtained by interpolation of the respective height ratios into the regression equation.

RESULTS AND DISCUSSION

Sample Preparation

The pH-solubility profile indicates that a neutral pH would be optimal for the extraction of I, although an adequate recovery is to be expected at any pH below 7.5. This substance is sparingly soluble in polar organic solvents (ethers, alcohols <75 mg/100 ml), and shows the greatest (non-aqueous) solubility in chloroform and dichloromethane (300-400 mg/100 ml). However, neither I nor the two metabolites, II and III, could be extracted from aqueous solution adequately with chloroform alone; the addition of isopropanol to the chloroform, however, improved the recovery of all three substances. The best extraction yields overall were obtained when the anionic ion pair reagent, sodium dodecyl sulphate, was added to the extraction solvent. Various mixtures of this counter ion with isopropyl acetate, isopropanol, methylene chloride, and chloroform were tried, and all variations gave good recoveries for all 5 substances (>70 %). The mixture chloroform/isopropanol/sodium dodecyl sulphate (9.8/4.2/1.0 [25 mM], v/v) was best (> 80 %), while substituting dichloromethane for chloroform gave

lower recoveries. The latter, however, was the method of choice because of the higher volatility and lower toxicity of dichloromethane compared with chloroform. As expected, the recoveries of the two most basic substances in this series, the N-demethyl metabolite and pipemidic acid, were the most improved by the addition of sodium dodecyl sulphate to the extraction solvent.

If only I has to be determined, then the ion pair reagent can be dispensed with in the extraction solvent; this has a practical advantage in that the presence of sodium dodecyl sulphate often leads to emulsion problems at the interface.

The influence of both pH and molarity of the ion pair reagent on the extraction was investigated. The recovery is at a maximum below pH 4 and then falls away as the pH is increased for all substances except the internal standard IV. Presumably, ion pair formation with sodium dodecyl sulphate is reduced as a result of suppression of the ionisation of the basic part of the molecule at higher pHs, although this does not explain the lack of pH dependency for IV.

With increasing concentrations of sodium dodecyl sulphate (SDS) up to 0.025 M, the recovery of all 5 substances increases rapidly to a maximum. Above 0.025 M SDS, the extractability falls slightly, this being associated with a noticeable increase in the tendency of the ion pair solution to foam.

Chromatography

Most of the reported methods for this class of substances indicated that the use of ion pair reagents in the mobile phase was essential to obtain acceptable peak shapes with minimum tailing on reversed phase columns. Tetrabutylammonium salts are commonly used, although heptane sulphonic acid was a successful alternative for the determination of the four quinolones, ciprofloxacin, norfloxacin, ofloxacin, and pefloxacin [7].

Several C_{18} reversed phase columns were tried for this method, including Nucleosil, Spherisorb, and μBondapak. The best efficiency and selectivity were obtained with the Toya Soda material; for the analysis

of I only in plasma, μBondapak was a reasonable alternative. As stated above, both anionic and cationic ion pair reagents have been used for this substance class, and we were also able to show that, for the analysis of I with IV as internal standard on μBondapak C_{18}, a mobile phase containing sodium octane sulphonate gave satisfactory chromatograms. The metabolites could not be separated under these conditions, however, so that tetrabutylammonium sulphate was the ion pair reagent of choice.

The proportion of methanol in the mobile phase was a critical factor for separation and analytical run time. A small decrease in methanol content (from 28 to 25.5 %) led to a 40 % increase in retention time (13 to 18 minutes at room temperature). The methanol content was especially critical for the separation between II and IV.

Detection

With UV detection at 290 nm a limit of quantification of 100 ng/ml is possible; for lower concentrations, fluorescence detection must be used and, depending on the quality of the column, a limit of 10-20 ng/ml is achievable (CV 4 %). For convenience of operation, fluorescence detection was routinely used; dilution of high concentration samples was necessary to bring these within the linear concentration ranges of the detector settings.

Choice of Internal Standard

Of the two internal standards used, the substance AM-735 (IV) resembles I most closely, differing only in having one fluorine atom less in the benzene ring. Pipemidic acid (V), which is readily available, is a more basic substance. This latter property is clearly indicated from the observation that the recovery of V from an aqueous solution is more improved by the addition of sodium dodecyl sulphate to the extraction solvent than is the recovery of I. Chromatographically, V is preferable since there are no separation problems and it is the earliest eluted component, so that the analysis is not delayed. Substance IV, on the other hand, is the last eluted component and, in addition, is only just

separated from the N-oxide metabolite II. Thus, any deterioration in the separation power of the column might be critical for the resolution of II and IV. However, for the analysis of plasma samples this is not important, since the metabolites are not detected. Either IV or V may be used as internal standard for plasma or urine analysis. Because of the closer structural similarity of IV to I than to V, IV is used for plasma analysis. For urine, because of the separation problems mentioned, pipemidic acid is used (see Figs. 2a and 2b for typical plasma and urine chromatograms).

Characteristics of the Method

Linearity

This parameter is detector dependent and must be tested for each fluorescence detector. For the Kontron SFM 23 on the "low" range setting used commonly for urine analysis the response is linear for up to 300 ng injected. For plasma samples, the "high" range is used and linearity is maintained for up to 100 ng injected. In fact, a weighted quadratic regression is used in our case, but only because a marginally better fit is obtained than with linear regression.

Extraction efficiency

A mean recovery of 81 % for I in plasma (concentration range 10 ng/ml to 5 μg/ml, method A), and 86 %, 81 %, and 80 % (10 to 100 μg/ml) for I, II, and III, respectively, in urine (method B) was found.

Precision and accuracy

The data are given in Table 1. Only parent substance has been detected in plasma and, in urine, the concentrations of the two metabolites are much lower than that of the parent substance. Thus, the somewhat lower precision for the metabolites may be mainly due to the lower concentrations tested. In particular, it was noticed that the N-oxide peak, with respect to tailing, was more affected by column deterioration than the other substances.

Limit of quantification

The stability of the fluorescence detector with respect to baseline noise would allow a detection limit of about 1 ng/ml. However, the

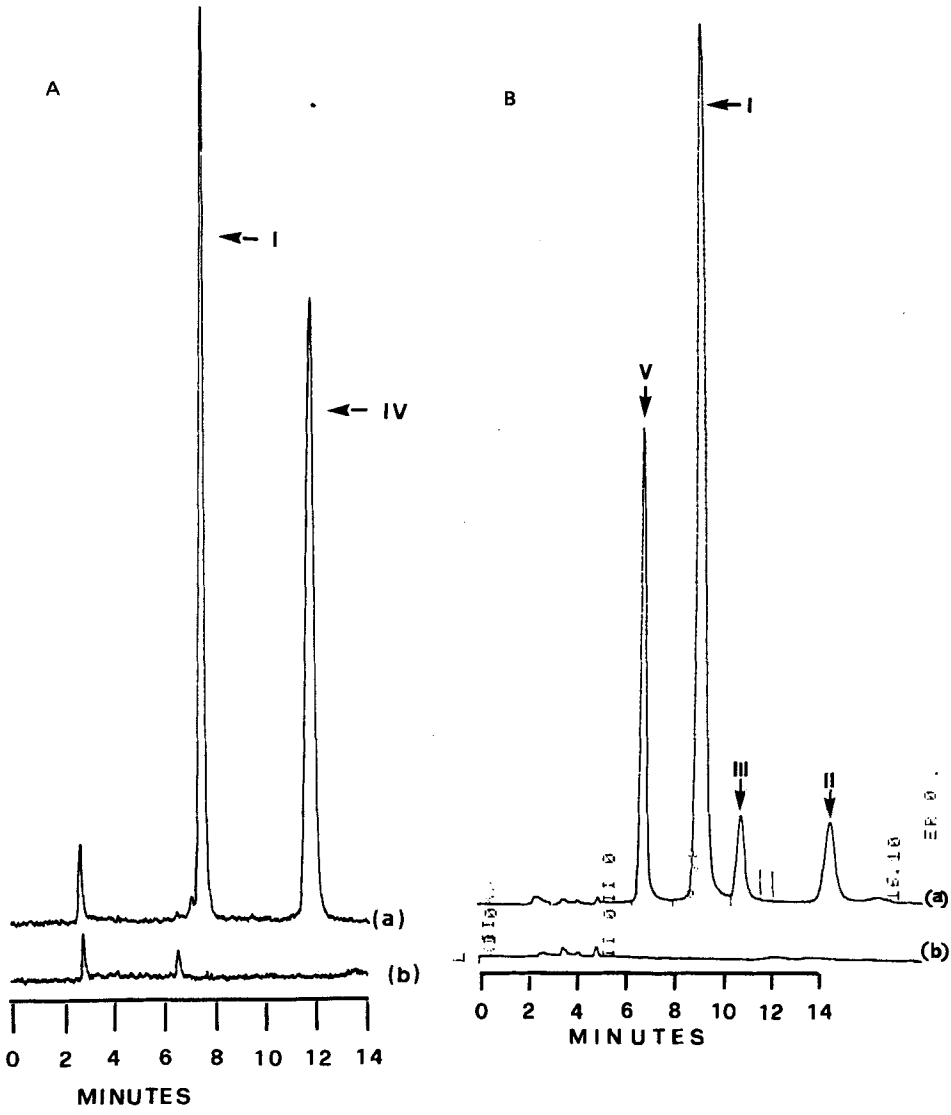


FIGURE 2 (a) Chromatogram (fluorescence detection) of a plasma sample from a subject 18 h after receiving a 200 mg oral dose of I (a). The peak (I) corresponds to a concentration of 626 ng/ml; (b) is the corresponding pre-dose sample.

(b) Chromatogram (fluorescence detection) of an 8-12 h urine sample from a subject after receiving a 400 mg oral dose of I (a). The peaks correspond to concentrations of 69, 15, and 15 μg/ml of I, II, and III, respectively; (b) is the corresponding pre-dose sample.

TABLE 1

Precision and Accuracy

A Plasma[a]

Concentration I added (μg/ml)	Mean concentration found	(n)	RSD	Error[b]
0.050	0.049	(7)	5.9	- 2
0.50	0.48	(7)	10.4	- 4
5.0	5.30	(10)	3.1	+ 6

B Urine (determined over a 3-week period)

Substance	Concentration added (μg/ml)	Mean concentration found (n = 12)	RSD	Error
I	40.0	39.8	4.0	- 0.5
	200.0	198.0	2.6	- 1
II	8.0	8.9	6.9	+ 11
	40.0	44.8	8.5	+ 12
III	8.0	8.2	9.7	+ 3
	40.0	40.3	4.8	+ 1

a All determined over a 4-week period, except for the 0.05 μg/ml sample which was over 1 week.
b Error, as defined as difference between found and added concentration, expressed as a percentage of the added amount.

presence of an endogenous component eluting just before I increases the quantification limit 10 to 20 ng/ml (RSD 3.6 %, n = 4, intra assay).

Stability

Using our established procedure for stability determinations [10], I, II, and III were found to be stable in human plasma and urine for at least 24 hours at room temperature, and at -20°C for 4 months (plasma) and 6 months (urine).

Quinolones are generally light sensitive, and this was investigated in the different types of vessels used during storage and analysis of

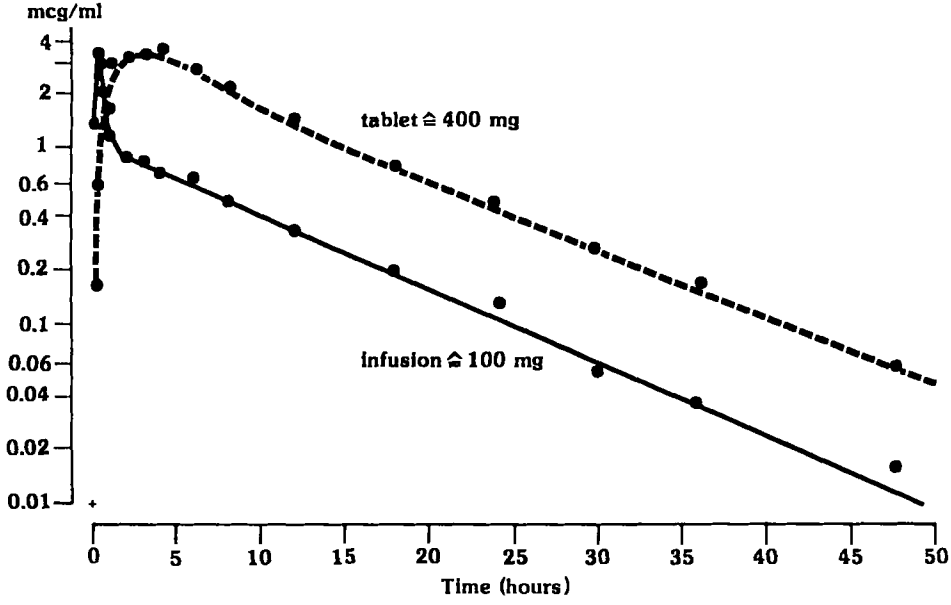


FIGURE 3 Time course of plasma concentration of I following oral (400 mg) and i.v. (100 mg) doses (single human subject).

samples. The results indicate that brown, and not clear glass vessels should be used to store plasma samples. However, urine is normally collected and stored in polypropylene bottles, and the results show that insufficient light is transmitted through this material to cause any degradation.

Specificity

The use of fluorescence as opposed to UV detection, whenever possible, always increases the selectivity of a method. Several thousand plasma and urine samples have been analysed, and no problems have yet been encountered with endogenous interferences. Possible interference from other quinolones was not investigated, since it is extremely unlikely that two or more of these substances woud be co-administered.

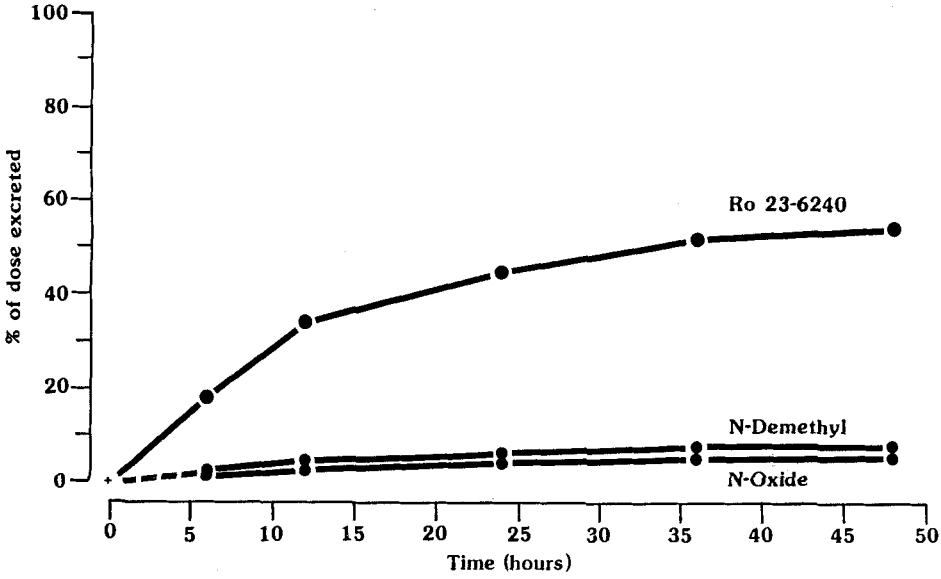


FIGURE 4 Cumulative urinary excretion of I, II, and III in man following oral administration (400 mg).

Application

As mentioned above, a large number of samples from human pharmacokinetic studies have been analysed. The average lifetime of these colums is about 2000 injections of biological fluid extracts. After this, the column flow direction can be reversed, whereby a further 200-300 injections of biological fluid are possible. Although not absolutely necessary, the use of a column heater (27°C) has the advantage of shortening the chromatographic elution time and maintaining stable retention times. The latter has proved to be an important point in connection with analysis taking place during both night and day because of room temperature variations during a 24 h-period. As an example of the application of this method, a plasma profile from a representative subject and an urinary excretion curve are shown in Figs. 3 and 4, respectively [11].

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