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DETECTION OF OXILOFRINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION AND COMPARISON WITH GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A high-performance liquid chromatographic (HPLC) method with electrochemical detection for the determination of oxilofrine [1-(4-hydroxyphenyl)-2-methylaminopropanol] in human plasma and urine (before and after cleavage of the metabolic conjugates) is described Isolation from biological fluids is performed batchwise by weak acid cation exchange. Separation of plasma and urine components is achieved on a reversed-phase C_{18} column as an ion pair with heptanesulphonic acid. For amperometric detection the potential of the electrode was set at 0.95 V versus an Ag/AgCl reference electrode The detection limit for oxilofrine in plasma is 1 ng/ml and in urine 12.5 ng/ml at a signal-to-noise ratio of 2.0 using 1.0 ml of plasma and 0.02 ml of urine. The method was compared with a gas chromatographic–mass spectrometric method and showed a good concordance for plasma ($r=0.996$) and urine ($r=0.994$). With the HPLC method it is also possible to determine related sympathomimetic drugs, e.g., etilefrine, norfenefrine or octopamine, after a slight modification of the mobile phase.

INTRODUCTION

Oxilofrine [1-(4-hydroxyphenyl)-2-methylaminopropanol] (Fig. 1) belongs to the group of monohydroxyphenylalkylamines, which are closely related to biogenic amines and act sympathomimetically, and specific analytical methods are required in order to isolate and separate these drugs from endogenous biological

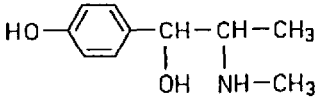

Fig. 1. Structure of oxilofrine

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material such as plasma and urine. The most specific procedure for the bioassay of sympathomimetic drugs so far is gas chromatography–mass spectrometry (GC–MS) [1,2]. However, for comprehensive pharmacokinetic studies GC–MS methods are usually too expensive and time-consuming.

Therefore, a specific high-performance liquid chromatographic (HPLC) method with electrochemical detection for the quantitation of both free oxilofrine in plasma and free and total oxilofrine in urine was developed in order to facilitate a broad pharmacokinetic study involving more than 1100 plasma and urine samples [3]. For validation of this method it was compared with a GC–MS method previously developed by us [2]. This paper describes the extraction and chromatographic conditions for the HPLC method and the comparison with GC–MS.

EXPERIMENTAL

Reagents

Oxilofrine hydrochloride (Albert Roussel, Wiesbaden, F.R.G.), *p*-tyramine hydrochloride, etilefrine hydrochloride, heptanesulphonic acid (HSS), Amberlite CG 50 cation-exchange resin (100–200 mesh), 98% (v/v) formic acid (Sigma, Munich, F.R.G.), EDTA, sodium acetate, distilled water for HPLC, potassium dihydrogenphosphate, potassium hydroxide (Merck, Darmstadt, F.R.G.) and methanol for HPLC (Roth, Karlsruhe, F.R.G.) were used. All reagents were of analytical-reagent grade and especially purified for HPLC use.

Apparatus

A Millipore–Waters Model M510 damped double-piston pump coupled with a Millipore–Waters WISP 710 autosampler and a Millipore–Waters Model 460 electrochemical detector was used. For peak integration a Hewlett-Packard Model 3390 integrator was connected.

HPLC was performed on a column (18.5 cm×4.6 mm I.D.) filled with RP Nucleosil C_{18} (5 μm), fitted with a pre-column filled with RP Nucleosil C_{18} (7 μm) (2.5 cm×4.6 mm I.D.) (Bischoff Analysentechnik, Leonberg, F.R.G.). The isocratic mobile phase contained 0.05 *M* sodium acetate, 50 mg/l EDTA and 100 mg/ml HSS, adjusted to pH 4.5 with acetic acid and mixed with 12.5% (v/v) methanol. The flow-rate was kept at 1.0 ml/min. The potential of the electrochemical detector cell was set at 0.95 V vs. an Ag/AgCl reference electrode.

Extraction of free oxilofrine from plasma

To 1.0 ml plasma in a 2-ml Eppendorf vial were added 10 μl of aqueous *p*-tyramine hydrochloride solution containing 1.0 mg/l *p*-tyramine base as internal standard, 0.2 ml of phosphate buffer (pH 7.4) and 40 mg of cation-exchange resin. The capped vial was shaken for 20 min on a Vortex mixer and centrifuged at 2000 *g* for 5 min at room temperature. The supernatant was discarded and the resin washed three times with 1.0 ml of 1% (v/v) methanol. To the resin was added 1.0 ml of 0.1 *M* formic acid and the mixture was shaken for 20 min on a Vortex mixer.

After centrifugation at 2000 *g* for 5 min at room temperature, an aliquot of the

supernatant was placed in a PTFE-lined capped vial for the WISP autosampler and 50 μl were injected on to the column.

Extraction of free and total oxilofrine from urine

Total oxilofrine (free and conjugated). To 20 μl of urine in a 1.5-ml glass vial were added 200 ng of etilefrine base as internal standard (20 μl of a 12 mg/l aqueous etilefrine·HCl solution), 0.2 ml of distilled water and 0.2 ml of 0.1 *M* hydrochloric acid. The vial then was closed with a PTFE-lined cap and heated at 100°C for 50 min. After cooling to room temperature, the contents of the glass tube were transferred into a 2-ml Eppendorf tube and 230 μl of 0.1 *M* potassium hydroxide solution, 1 ml of phosphate buffer (pH 7.4) and 40 mg of cation-exchange resin were added. All further extraction steps were identical with those in the plasma method described above.

Free and unchanged oxilofrine. To 20 μl of urine in a 2-ml Eppendorf tube were added 200 ng of etilefrine base as internal standard (20 μl of the 12 mg/l aqueous etilefrine·HCl solution), 1 ml of phosphate buffer (pH 7.4) and 40 mg of cation-exchange resin. All further extraction steps were identical with those in the plasma method described above.

Calibration for the quantification of oxilofrine in plasma and urine

Four calibration graphs were needed for the quantification of oxilofrine in plasma, urine and hydrolysed urine. Calibration samples were prepared by spiking plasma and urine samples with oxilofrine and the samples were then extracted according to the methods described above. The peak-height ratio (oxilofrine to internal standard) was plotted against concentration. The calibration ranges selected were as follows: for plasma, (a) 1.0–10.0 ng/ml and (b) 10.0–200.0 ng/ml; for urine (free), (c) 25–500 ng per 20 μl urine; and for urine (total), (d) 50–1000 ng per 20 μl. The equations for the regression lines and the correlation coefficients were (a) $y=0.046x-0.0003$, $r=0.997$; (b) $y=0.057x-0.113$, $r=0.998$; (c) $y=0.008x-0.220$, $r=0.997$; and (d) $y=0.0074x-0.058$, $r=0.995$.

RESULTS AND DISCUSSION

Chromatography

With the described method it is possible to measure oxilofrine in both plasma and urine selectively and rapidly. The drug is separated well from endogenous compounds, i.e., biogenic amines, which are isolated by the ion-exchange resin (Figs. 2 and 3) too.

No interferences were observed from norepinephrine, epinephrine or dopamine in plasma or urine. The reason for using different internal standards for plasma and urine (*p*-tyramine and etilefrine, respectively) is that *p*-tyramine occurs in urine in relatively large amounts [4], whereas in plasma it is not detected under the given conditions. Normal *p*-tyramine concentrations in human plasma have been found to be 0.68 ± 0.09 ng/ml [5]. The plasma *p*-tyramine level is considerably increased after oral intake of *p*-tyramine in the first 2 h after administration [6], but this method is primarly orientated to pharmacokinetic and

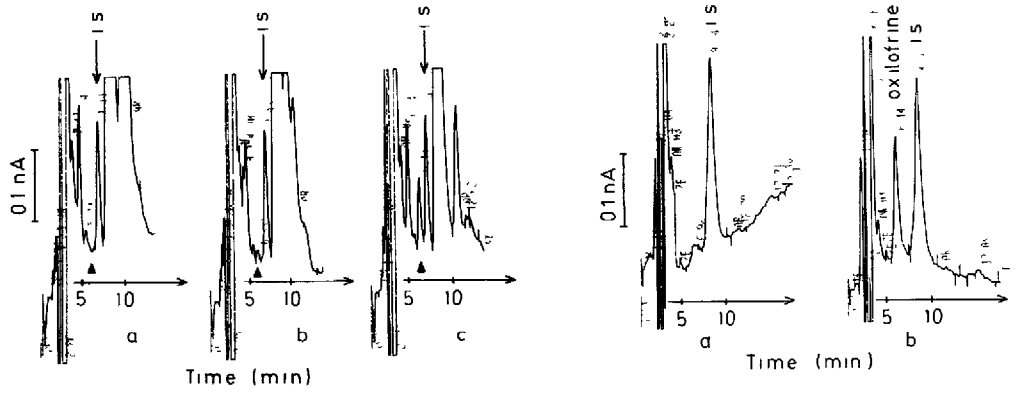

Fig. 2. Chromatograms of a volunteer's plasma extract: (a) without oxilofrine and (b) with 1.5 ng/ml and (c) with 10.3 ng/ml oxilofrine (▲). Retention times: oxilofrine = 6.44 min; *p*-tyramine (internal standard, i.s.) = 7.47 min

Fig. 3. Chromatograms of a volunteer's urine extract: (a) without oxilofrine and (b) with 6.6 μg/ml oxilofrine. Retention time of etilefrine (i.s.) = 9.36 min.

pharmacodynamic studies with volunteers or patients who have to maintain a special diet free from sympathomimetically acting compounds. *p*-Tyramine was found to be more suitable as an internal standard for the plasma analysis by HPLC in the low detection range of 1–200 ng/ml than etilefrine, which was used in the GC–MS method [2].

Electrochemical detection

In contrast to the low potential required for the detection of catechol compounds, the oxidation of monohydroxyphenyl compounds demands higher voltages. The selected electrode potential of 0.95 V vs. Ag/AgCl was found to be a good compromise between sensitivity of detection and suppression of biological background.

Precision, recovery and detection limit

The intra-assay coefficients of variation were determined by measuring plasma and urine samples (the latter before and after acid hydrolysis) from several volunteers, and were found to be 3.5% for plasma, 2.0% for urine and 6.4% for hydrolysed urine.

The inter-assay coefficients of variation were found to be 8.1% for plasma and 6.6% for urine.

Validation of the calibration graphs was performed by measuring a plasma or urine sample spiked with a known concentration of oxilofrine in every series.

Recovery

The recovery of oxilofrine from plasma and urine (the latter before and after acid hydrolysis) was determined by comparing the peak counts of oxilofrine after extraction with those of a standard oxilofrine solution with the corresponding

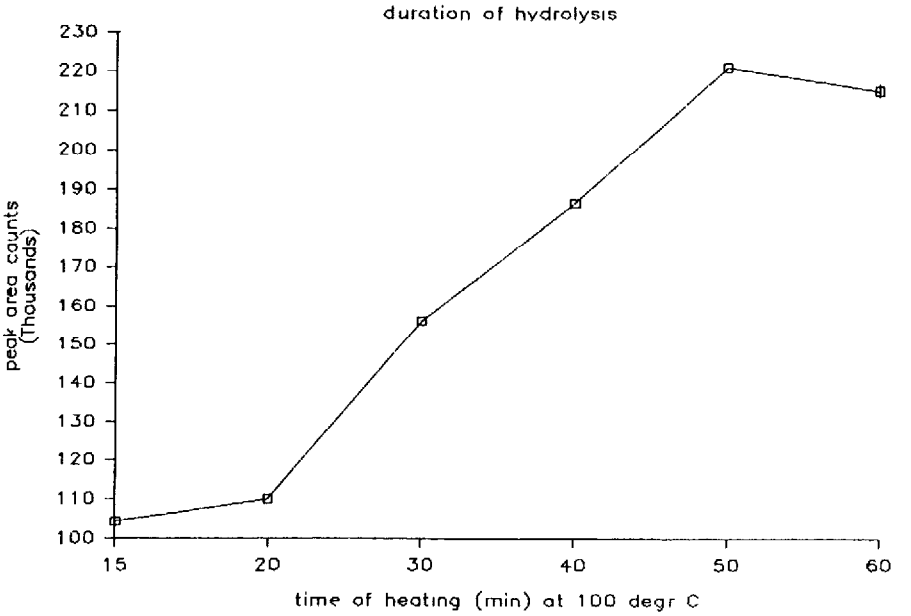

Fig. 4. Rate of deconjugation versus time of heating at pH 1.

range of concentrations. The mean recovery of oxilofrine from plasma was 70%, from urine 50% and from hydrolysed urine 42% ($n=7$).

Hydrolysis

The rate of deconjugation of the oxilofrine sulphate and glucuronide in urine by acid hydrolysis depends on the temperature, pH and time of heating. The optimal rate of deconjugation was tested by varying each of the parameters. However, we could only determine the maximal degree of deconjugation by measuring urine samples with unknown amounts of total oxilofrine, because we had no oxilofrine conjugate standard at our disposal. The optimal temperature and time of heating were those with the minimal degree of decomposition of the drug and the maximal rate of deconjugation (Fig. 4).

Using the above-described volumes of plasma and urine the detection limit for oxilofrine in plasma was 1 ng/ml and in urine 0.25 ng per 20 μl ($=12.5$ ng/ml) at a signal-to-noise ratio of 2.0. The reason for the chosen volumes was the use of the Eppendorff tubes and mini glass vials.

Validation of the HPLC method by comparison with GC–MS (accuracy)

The HPLC method was compared with our GC–MS method [2], in which the isolation of oxilofrine from plasma and urine is common to that of the HPLC method up to the elution step from the ion-exchange resin. In the HPLC method elution is performed with formic acid whereas in the GC–MS method a simultaneous extraction–derivatization step is used [7,8].

For comparison of the two methods, plasma and urine samples ($n=49$) derived from the above-mentioned pharmacokinetic study [3] were analysed and the

TABLE I

OXILOFRINE CONCENTRATIONS IN A VOLUNTEER'S PLASMA AND URINE EXTRACTS, THE LATTER BEFORE AND AFTER ACID HYDROLYSIS

No.	Sample*	Concentration**		Pair difference, GC-MS – HPLC (ng/ml)
		GC-MS	HPLC	
1	P	0 00	0.00	0.00
2	P	0 00	0.00	0.00
3	P	1 07	0.89	0.18
4	P	1.49	1.80	−0.31
5	P	2.15	1.80	0.35
6	P	2.23	2.10	0.13
7	P	3.55	3.85	−0.30
8	P	5.11	5.14	−0.03
9	P	5.27	5.70	−0.43
10	P	5.62	5.94	−0.32
11	P	6.14	6.00	0.14
12	P	6.61	7.30	−0.69
13	P	7.14	6.97	0.17
14	P	7.19	7.21	−0.02
15	P	7.39	7.03	0.36
16	P	8.40	8.70	−0.30
17	P	9.43	9.12	0 31
18	P	9.66	9.95	−0.29
19	P	9.81	9.38	0.43
20	P	15.32	16.79	−1.47
21	P	16.29	16.81	−0.52
22	P	22.57	19.71	2.86
23	P	30.96	30.44	0.52
24	P	37.77	38.80	−1.03
25	P	44.06	41.47	2.59
26	P	64.65	65.90	−1.25
27	P	85 99	92.20	−6.21
28	P	93 90	93.55	0.35
29	P	131.10	126.90	4.20
30	P	137.88	130.00	7.88
31	P	213.62	238.96	−25.34
1	U	0.00	0.00	0.00
2	U	0.00	0.00	0.00
3	U	21.92	22.05	−0.13
4	U	28.27	19.83	8.44
5	U	30.90	27.57	3.33
6	U	41.99	42.95	−0.96
7	U	155.89	198.82	−42.93
8	U	443.00	464.87	−21.87
9	U-hy	12.73	14.42	−1.69
10	U-hy	17.68	21.97	−4.29
11	U-hy	21.66	28.04	−6.38
12	U-hy	27.89	21.32	6.57
13	U-hy	162.50	149.99	12 51
14	U-hy	169.97	150.57	19 40
15	U-hy	214.30	206.36	7.94
16	U-hy	278.02	226.16	51.86
17	U-hy	588.70	587.09	1.61
18	U-hy	874.40	835.77	38.63

*P = plasma; U = urine; U-hy = hydrolysed urine.

**Concentrations: ng/ml in plasma, ng per 20 μl in urine.

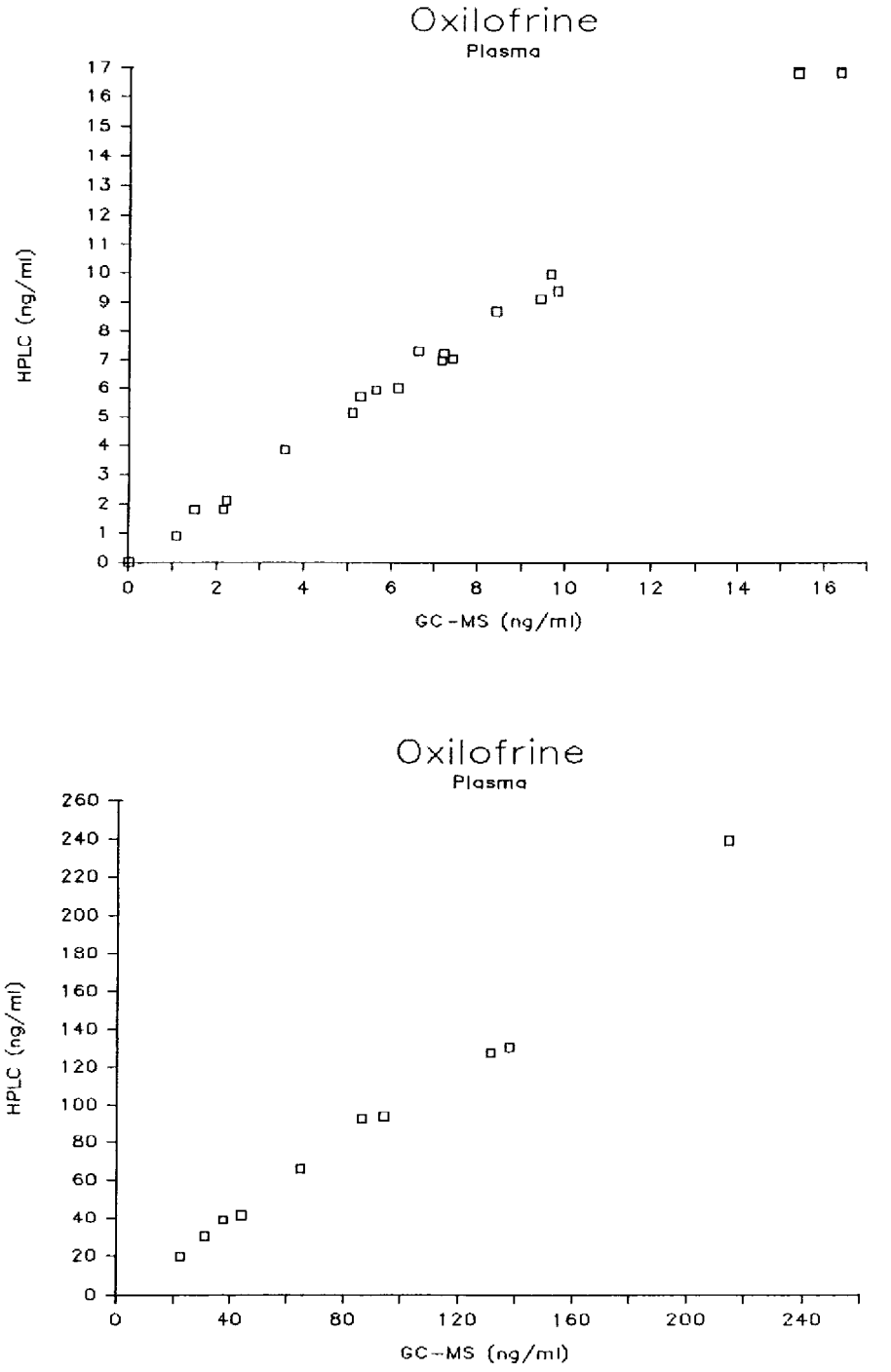

Fig. 5. Concentrations of oxilofrine in plasma determined by HPLC versus GC–MS in the ranges 0–20 ng/ml (top) and 20–240 ng/ml (bottom).

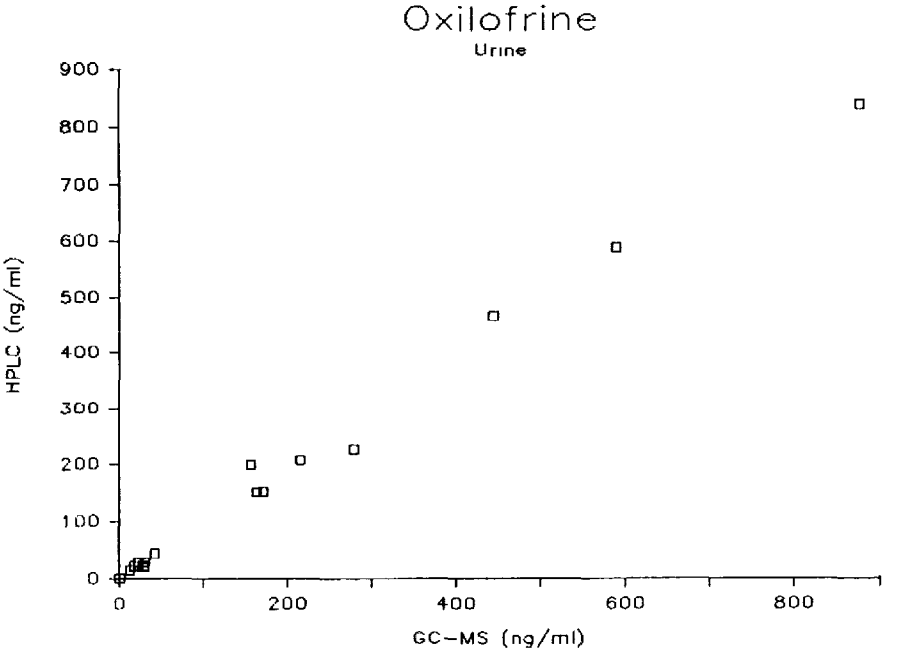

Fig. 6. Concentrations of oxilofrine in urine determined by HPLC versus GC–MS.

concentrations determined by HPLC were plotted against those obtained by GC–MS. In Table I the plasma and urine concentrations of oxilofrine obtained by each method are given.

The oxilofrine plasma levels are plotted in Fig. 5 and show a good correlation in the lower and upper concentration ranges ($r=0.996$, 0.994). For urine the correlation between the methods is also good ($r=0.996$) (Fig. 6).

Plotting the difference (GC–MS minus HPLC as a percentage of the HPLC concentrations) against the concentrations in one method showed (Fig. 7) that 87% of all pairs of values deviate by less than 10% from each other. In urine (Fig. 7) these differences in the pairs of values are larger but still acceptable; here 81.3% of all values are within a 20% deviation from one of the methods. The nearly equal distribution of positive and negative differences for both plasma and urine shows that the HPLC method gives comparable results to those given by GC–MS.

Applicability of the HPLC method

Using the proposed HPLC method a comprehensive pharmacokinetic study was performed, investigating the relative bioequivalence of different formulations and dosages of oxilofrine (Carnigen^{TM}) in twelve healthy subjects. In Fig. 8 the plasma concentration-time curves of 16-, 32- and 64-mg dragée dosages compared with a 16-mg liquid formulation as a reference dosage are shown. For pharmacokinetic details see ref. 3.

The described method can be easily adapted for the determination of other

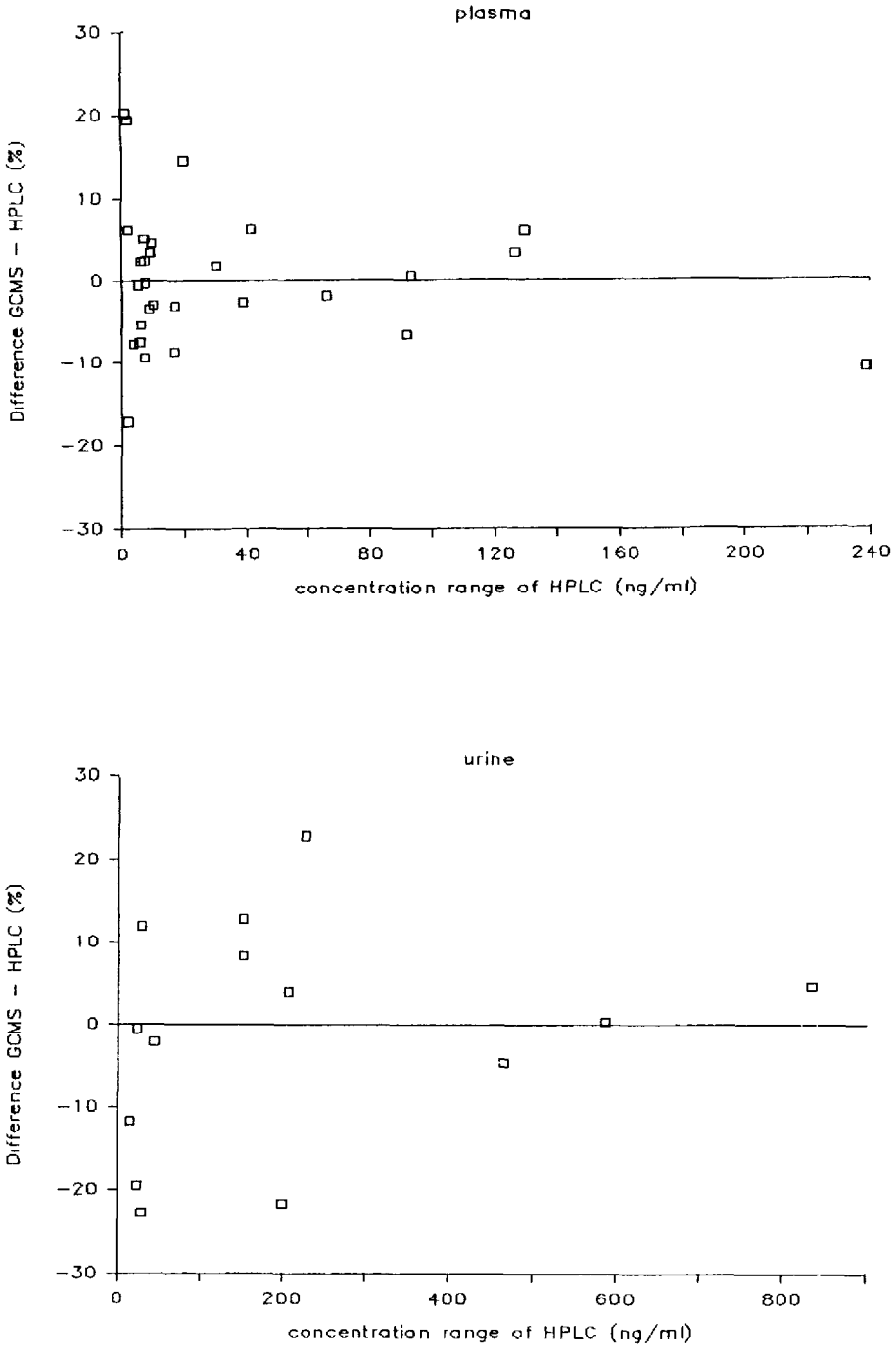

Fig. 7. Relative deviation of concentrations of oxilofrine in plasma and urine obtained by HPLC and GC-MS. (Pair difference GC-MS minus HPLC related to the concentrations measured by HPLC.)

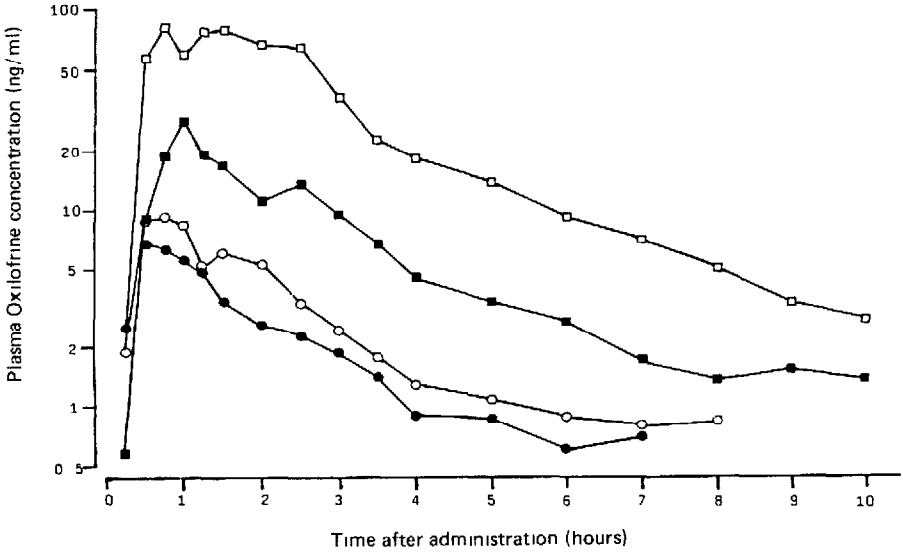

Fig. 8. Plasma concentration–time curves (mean curves for twelve subjects) of oxilofrine obtained after different doses of oxilofrine: ●, 16-mg drops; ○, 16-mg dragée; ■, 32-mg dragée; □, 64-mg dragée.

sympathomimetic drugs, e.g., etilefrine, norfenefrine or octopamine. For these compounds only slight modifications of the composition of the mobile phase are required.

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