Quantitation of Urapidil and its Metabolites in Human Serum by High Performance Liquid Chromatography

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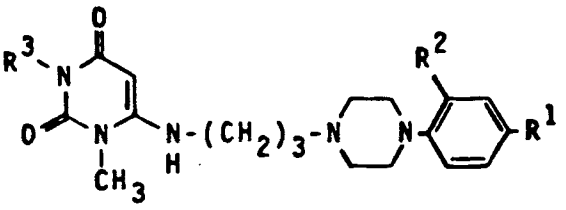
Summary

The antihypertensive agent urapidil (Ebrantil Byk-Gulden, Konstanz) can be reliably quantitated with three metabolites in human serum using high performance liquid chromatography. Serum is alkalinized and extracted with ethyl acetate. The organic phase is back-extracted with diluted acid. An aliquot is sampled automatically and chromatographed in an optimized combination of mobile and stationary phase. UV-detection at 273 nm allows a quantitation limit of 5 ng/ml for all analytes. Precise handling of exact volumes facilitates external calibration. The coefficient of variation for spiked samples is less than 5% within and less than 7% between studies. Application of the method to experimental and clinical pharmacokinetic studies of urapidil is illustrated.

1 Introduction

Urapidil (Ebrantil) is a substituted phenylpiperzine derivative which was synthesized by *Klemm* and *Prüsse* [2] in the Research Laboratories of Byk Gulden Pharmaceuticals in Konstanz, Germany. It has remarkable antihypertensive properties. In Germany the intravenous form was introduced in 1980, the oral form in 1981. The usual dosage is 30 to 90 mg/day for oral treatment of hypertension, and 25 or 50 mg, single or multiple, for hypertensive crises [3]. Drug disposition in man was summarized recently [1]. After intraveneous administration a biphasic serum level profile is observed with a half-life in the α-phase of approximately three hours. After oral administration the maximum serum level is reached after three hours while the apparent half-life of elimination is five hours.

In the body, urapidil is metabolized in several ways (**Figure 1**): *p*-hydroxylation (M1), *O*-demethylation of the methoxyphenyl ring (M2) and *N*(1)-demethylation of the uracil residue (M3) [4]. In man, *p*-hydroxylation is the preferred metabolization; the pharmacological activity of M1 *in vitro* is approximately two orders of magnitude less than that of urapidil [7].



R^1	R^2	R^3	Name
OH	OCH_3	CH_3	M1
H	OH	CH_3	M2
H	OCH_3	H	M3
H	OCH_3	CH_3	Urapidil

Figure 1

Structures of urapidil and its metabolites.

Several assay methods for quantitation of urapidil and metabolites in body fluids have been reported [4-8]. The small dosages result in low body fluid concentrations of urapidil and its metabolites. High performance liquid chromatography is the method of choice for analysis, although the sensitivity was not entirely adequate, especially for quantitations in the low nanogram range. An improved MS method [6] with direct sample introduction facilitated reliable quantitation down to 20 ng/ml.

2 Experimental

2.1 Materials

Pure standards of urapidil and the metabolites M1, M2, and M3 were kindly provided by Byk-Gulden, Konstanz. All other reagents, analytical or HPLC-grade, were purchased

Table 1

Recoveries of urapidil and M1 from serum.

Spiked conc. in serum (ng/ml)	Recovery urapidil (%)	Recovery M1 (%)
5	90	75
10	93	73
25	92	72
50	95	74
100	95	74
200	94	73
400	93	73
Mean	93	73
Sdev	1.0	1.2
cv:	1.1	1.3

from E. Merck (Darmstadt) and used without further purification. Mobile phase components (water, acetonitrile) were separately filtered prior to mixing, then degassed after mixing.

2.2 Apparatus and Chromatographic Conditions

An automated HPLC-chromatograph consisting of modular components was used. Components were a Beckman 112 solvent delivery pump, a Kontron model 730 variable wavelength spectrophotometer operated at 273 nm, and a Waters model 710 B automatic sample processor. Detector output (peak area) was quantitated using a Shimadzu CR 1 B integrator. Data regression and concentration calculations were performed on a Commodore CBM 8032 Computer using the LAB CAL software program.

The column was a Merck LiChrosorb CN, 5 μm, 125 × 4 mm. The mobile phase consisted of water (1300 g), acetonitrile (250 g), and PIC B 6 reagent (one ready-to-use bottle). The flow rate was 1.0 ml/min. All analyses were performed at room temperature.

2.3 Stock Solutions

Standard solutions of urapidil and metabolite M1 were prepared by dissolving 100 mg of each compound in 100 ml methanol. Working solutions were prepared by appropriate dilution with water. Solutions are stable for at least three months when stored at 4°C in a freezer.

2.4 Preparation of Samples

The described sequence has to be followed exactly, otherwise the results are not reproducible. 1.00 ml serum, 20 μl of a 5% EDTA solution, 100 μl 0.1 N NaOH and 6.00 ml ethyl acetate were added to 10 ml round bottom culture tubes equipped with PTFE-lined screw-top caps. The samples were gently shaken in the upright position in a Vortex mixer for 60 s. After centrifuging at 2000 rpm, exactly 5.00 ml of the organic phase was transferred to a 10 ml tapered glass tube filled with 100 μl 0.1 N HCl. The tube was capped with a polyethylene stopper and shaken gently on a Vortex mixer for 30 s. After centrifuging at 2000 rpm, the organic phase was aspirated. The aqueous phase was transferred to an automatic sampling vial with a limited volume insert. The automatic sampler was programmed to inject 20 μl of each sample. For a concentration of 5 ng/ml the absolute amount of detected substance is less than 1 ng. The signal-to-noise ratio under these circumstances is usually greater than 10.

3 Results

3.1 Evaluation of the Method

The analytes showed critical elution behavior in different chromatographic systems. Especially urapidil has a marked tendency to tailing like many other basic compounds. The conventional techniques used to overcome this problem, *e.g.* ionic suppression or ionic partition, failed. The ion-pairing technique showed better results; for a final optimization of the peak shapes an extensive search for the best stationary phase was necessary. As a general rule, the longer the alkyl chain bound to silica, the more peaks tailed at the same capacity factors. Optimal were materials with short alkyl sites or nitrile functions. Together with a ternary mobile phase isocratic separation of all four analytes from one another and from endogeneous interference was achieved in a total analysis time of ten minutes.

Fluorescence detection, electrochemical detection, and UV detection at different wavelengths were tried. We found UV detection at 273 nm superior to all other methods in terms of sensitivity, safety of operation, long-time stability, and reliability. The limit of quantitation of 5 ng/ml serum for urapidil and M1 can be kept reproducible with small standard deviations and is sufficient for monitoring small single doses.

The back-extraction procedure allows high sample throughput and delivers an almost interference-free concentrate. The recovery rates for urapidil and M1 from 5 ng/ml to 1000 ng/ml with our method are 93% and 73% respectively, details are given in **Table 1**. The linearity of the method was checked up to concentrations of 800 ng/ml, the correlation coefficients remained better than 0.995 for each assay sequence and both analytes.

3.2 Statistical Evaluation of the Assay Quality

The unknown concentrations of the samples are quantitated by comparison with the peak areas of samples spiked with known concentrations. We used external calibration, as the available internal standard coeluted with urapidil and the analytes showed consistent recovery over the used range. To validate the reliability of external standard

Table 2

R^2 slopes and intercepts of urapidil calibrations of one study. The residuum is calculated after upweighted regression.

Cal-No.	R^2	Slope	Intercept
1	0.9992	590.3	-1038.9
2	0.9997	585.7	- 444.1
3	0.9998	601.6	-1322.4
4	0.9992	579.7	- 791.9
5	0.9998	598.5	- 708.4
6	0.9999	569.0	-1242.3
7	0.9998	570.0	- 282.4
8	0.9998	568.6	- 247.1

Table 4

R^2 slopes and intercepts of M1 calibrations of one study. The residuum is calculated after unweighted regression.

Cal-No.	R^2	Slope	Intercept
1	0.9992	359.8	-228.2
2	0.9990	351.7	341.9
3	0.9980	358.1	329.1
4	0.9990	359.2	47.5
5	0.9985	369.6	-283.5
6	0.9998	356.5	-286.6
7	0.9991	355.4	252.0
8	0.9993	353.4	-155.8

methods, a careful statistical control of the calibration has to be done. **Table 2** shows the results of eight calibration sequences with spiked urapidil concentrations from 5 ng/ml to 400 ng/ml. The peak areas are processed by 1/x weighting; from the weighted results the slope and intercept of the calibration curve are determined. This procedure is useful for wide-range calibrations.

Table 3 presents the mean, standard deviation, accuracy, and precision over all eight calibrations and all spiked concentrations for urapidil. At the limit of quantitation the precision is 8.2% with an accuracy of 0.3%, at higher concentrations the precision improves to less than 2% with a typical accuracy of better than 5%.

Table 4 shows the results of eight calibration sequences with spiked M1 concentrations from 5 ng/ml to 200 ng/ml. The peak areas are processed in the same manner as for urapidil.

Table 5 presents the results of mean, standard deviation, accuracy, and precision over all eight calibrations and all spiked concentrations for M1. At the limit of quantitation the precision is 9.3% with an accuracy of 2.1%, at higher concentrations the precision improves to less than 2% with a typical accuracy of better than 3%.

As the 1/x weighting can produce different results compared with the unweighted calculations, the above results are compared with the results of previous studies calculated unweighted. In these studies the calibration of both urapidil and M1 ranged from 5 to 100 ng/ml. **Table 6** presents the slopes of all calibration curves for urapidil from two studies which were three months apart. The mean of all slopes vary about 6% within and 6.4% between studies. The regression coefficients of all curves are better than 0.995, the intercepts equal 1 ng/ml.

Table 3
Statistical data for urapidil calibration.

		Concentration (spiked) (ng/ml)						
		5.00	10.0	25.0	50.0	100	200	400
Cal. curve	Regr. model	Concentration (found) (ng/ml)						
1	2	5.10	9.58	24.4	50.0	104	206	391
2	2	5.37	10.2	23.8	46.1	102	204	399
3	2	4.67	9.65	24.6	51.9	108	207	384
4	2	4.63	11.1	24.2	47.4	104	206	393
5	2	4.82	9.63	23.5	53.5	106	208	384
6	2	5.79	9.40	22.7	49.5	99.6	199	403
7	2	5.10	9.58	25.3	49.4	103	199	399
8	2	4.64	11.3	23.6	49.3	103	198	400
Number		8.00	8.00	8.00	8.00	8.00	8.00	8.00
Mean		5.02	10.0	24.0	49.6	104	203	394
sdev		0.413	0.728	0.784	2.33	2.68	4.07	7.34
Accuracy (%)		0.308	0.434	-4.0	-0.73	3.71	1.67	-1.4
Precision (%)		8.23	7.25	3.27	4.69	2.58	2.00	1.86

Table 5

Statistical data for M1 calibrations.

Cal. curve	Regr. model	Concentration (spiked) (ng/ml) 5.00	12.5	25.0	50.0	100	200
		Concentration (found) (ng/ml)					
1	2	5.28	12.3	23.9	48.5	104	198
2	2	5.57	10.3	28.1	49.7	104	197
3	2	4.59	13.1	28.0	47.9	106	195
4	2	4.58	12.6	25.3	53.4	102	192
5	2	4.18	12.9	27.9	50.7	103	193
6	2	5.27	12.0	25.3	49.1	98.5	202
7	2	4.80	13.1	–	51.1	96.1	202
8	2	4.88	12.1	25.3	53.7	97.6	198
Number		8.00	8.00	7.00	8.00	8.00	8.00
Mean		4.89	12.3	25.7	50.5	101	198
Sdev		0.46	0.91	1.21	2.18	3.56	3.48
Accuracy (%)		−2.1	−1.6	2.74	1.01	1.46	−1.1
Precision (%)		9.32	7.40	4.72	4.27	3.51	1.78

Table 6

Comparison of two independent studies on urapidil.

	Study A (Jan. 84)				Study B (April 84)		
Cal-No.	Slope	Intercept	R^2	Cal-No.	Slope	Intercept	R^2
1	565	−1012	0.9993	1	590	−1038	0.9990
2	573	214	0.9996	2	585	− 444	0.9987
3	571	− 972	0.9979	3	601	−1322	0.9992
4	588	− 674	0.9992	4	580	− 791	0.9988
5	602	− 900	0.9965	5	599	− 708	0.9990
6	593	−1106	0.9994	6	569	−1242	0.9989
7	573	− 512	0.9981	7	570	− 282	0.9978
8	582	− 422	0.9990	8	569	− 247	0.9997
Mean:	581	− 673	0.9986		583	− 759	0.9989
Sdev:	12.6	434	0.001		13.1	418	0.005
cv:	2.17	64	0.106		2.25	55	.05

Table 7

Comparison of two independent studies on M1.

	Study A (Jan. 84)				Study B (April 84)		
Cal-No.	Slope	Intercept	R^2	Cal-No.	Slope	Intercept	R^2
1	360	−228	0.9993	1	318	498	0.9989
2	352	342	0.9990	2	294	88	0.9991
3	358	329	0.9980	3	331	572	0.9990
4	359	48	0.9990	4	306	170	0.9989
5	370	−284	0.9985	5	300	122	0.9986
6	357	−286	0.9998	6	292	360	0.9979
7	355	252	0.9981	7	300	287	0.9990
8	353	−155	0.9993	8	307	280	0.9990
Mean:	358	2.25	0.9989		306	297	0.9988
Sdev:	5.6	275	0.0006		13.0	173.5	0.0005
cv:	1.57	107	0.063		2.25	58	.05

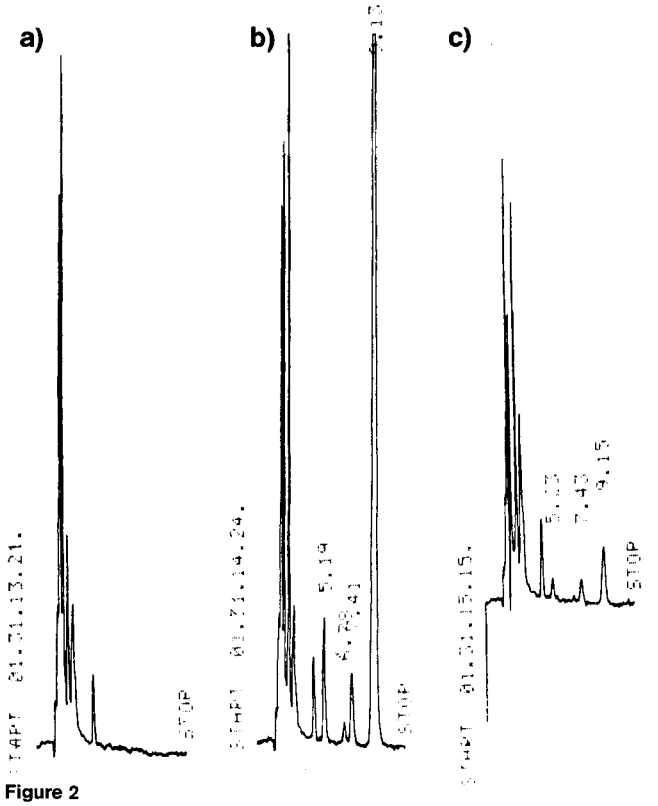


Figure 2

a) Chromatogram of a subject's predose sample; b) Chromatogram of the subject's 3 h sample; peak at 5.19 is about 25 ng/ml M1, peaks at 6.78 and 7.41 are M2 and M3, peak at 9.13 is about 210 ng/ml urapidil; c) Chromatogram of the subject's 12 h samples; peak at 5.23 is about 6 ng/ml M1, peak at 7.43 is M3, peak at 9.15 is about 15 ng/ml urapidil.

Table 7 shows the slopes of all calibration curves for M1 from two studies which were three months apart. The mean of all slopes varies about 7.2% within and 7.5% between studies. The regression coefficients of all curves are better than 0.995, the intercepts equal 0.8 ng/ml.

The calibration and the resulting quantitations are not influenced by the mode of calculation: the results of temporally different studies are consistent.

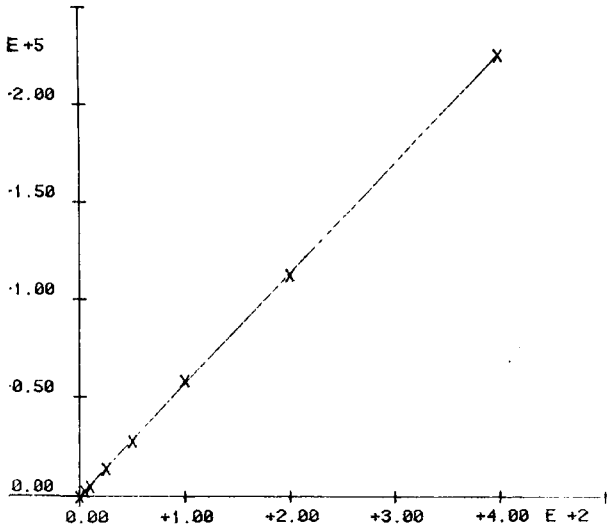
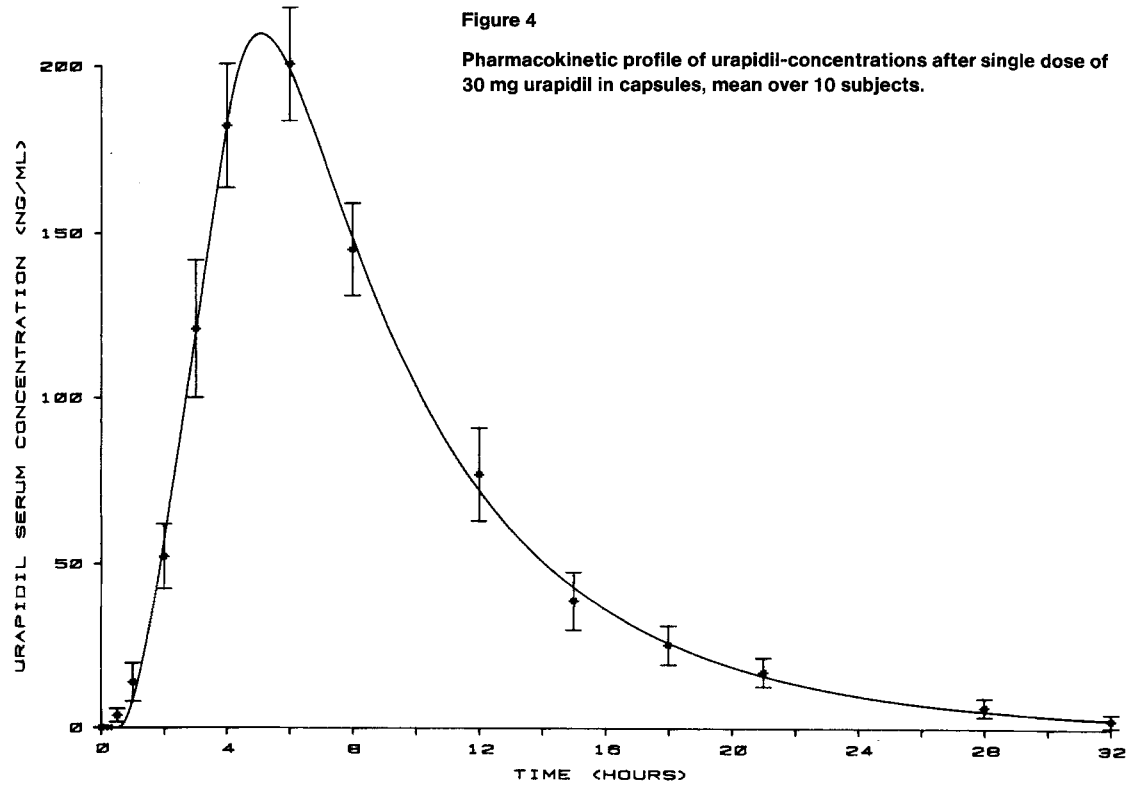


Figure 3

Typical calibration curve of urapidil, concentrations not weighted.

Figure 4

Pharmacokinetic profile of urapidil-concentrations after single dose of 30 mg urapidil in capsules, mean over 10 subjects.



3.3 Application to Human Studies

With the described method more than 3000 samples from several different studies have been measured. The results from a study with ten healthy volunteers serve as an example. Each volunteer was administered a single dose of 30 mg urapidil in capsules. **Figure 4** shows the mean curve of the measured serum concentrations from subjects with standard error. For 30 mg capsules given with breakfast, Cmax was about 200 ng/ml at a Tmax of 5.3 hours. The concentrations of M1 reached only one tenth of the urapidil concentrations, M2 and M3 are usually below the limit of quantitation. The occurrence of peaks as in **Figure 2b** is an exception. These metabolites were not quantitated.

4 Discussion

This paper describes a rapid and sensitive HPLC-method for quantitation of urapidil and metabolites in human serum. The analytes are extracted from alkalinized serum into organic solvent and back-extracted into diluted acid. The extracts are automatically sampled. The isocratic chromatographic system is optimized in terms of peak symmetry and chromatographic resolution. The limit of quantitation is sufficient for essentially any type of basic or clinical pharmacokinetic study of urapidil.

The external calibration procedure gives precise, reproducible, and accurate results within and between temporally different studies, as demonstrated by statistical data. After a single dosage of 30 mg urapidil in capsules the range of the 10 individual Cmax-values was 138-273 ng/ml; the Tmax-values ranged from 3 to 6 hours. The metabolites were only present in minor concentrations of less than 5% of the parent compound.

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