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Method development for dihydralazine with HPLC-MS/MS—an old but tricky substance in human plasma

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Abstract

An HPLC-MS/MS method was developed and validated for the determination of dihydralazine in human plasma. HPLC-MS/MS has not been used before in a published paper and provides better sensitivity and selectivity. Therefore a much easier sample preparation than published before is feasible (protein precipitation). As this substance is rather reactive and sensitive some specific care has to be taken hindering the conversion of the substance in whole blood and following human plasma after blood withdrawal. Hydrazines often are used for derivatization of aldehydes and ketones. With specific care (using 1,4-dithiothreitol (DTT) and cooling) dihydralazine can be preserved and analysed without decomposition or conversion in the tested range of 0.500–302 ng/mL of human plasma. The following inter-batch precision and accuracy of the Quality Control Samples resulted: QC-A (1.34 ng/mL plasma) with a precision of coefficient of variation (CV) 7.66% and an accuracy of 103.2%; QC-B (18.2 ng/mL 7.86%, acc. 101.3%); QC-C (258 ng/mL, 9.73%, acc. 98.3%). The inter-batch values of the LLOQ samples at 0.500 ng/mL were 7.17% for CV and accuracy of 106.4%. Mean recovery tested at the QC levels was found to be 103.8%. Specificity in six different plasma samples was good (<10% of the area of the LLOQ). Stability in plasma was tested under different conditions and was sufficient.

Keywords: Dihydralazine; Human plasma; Liquid chromatography mass spectrometry; Degradation of dihydralazine; 1,4-Dithiothreitol (DTT) as stabilizer

1. Introduction

Dihydralazine is an old substance in the therapy of hypertension. In the time after market introduction, few methods for the determination were published—all of them around 1980 [1–5]. These methods encountered difficulties determining unchanged and so-called "apparent" dihydralazine. This apparent dihydralazine means the lost dihydralazine in human plasma during plasma gaining from whole blood and during sample storage under freezing conditions and thawing before stopping the process of reaction or destroying. This means substances form the same derivative after some reactions like dihydralazine. In our testing of the described procedures [1–4] for stopping that process and for releasing the bound part of dihydralazine we found that this was not possible and not reproducible. Rouan and Campestrini [1] used 3 M HCl heating for 30 min at 90 °C and a derivatization step with HNO_2 and sodium methoxide afterwards. Siegmund et al. [2] used acetylacetone for derivatization and a short time for plasma sample gaining.

Degen et al. [3] used 1.5 N sulfuric acid and $NaNO_2$ heating for 25 min at 90 °C for releasing acid labile compounds and ring formation. In second step another derivatization with sodium methylate formed a stable derivative.

Waller et al. [4,5] used 2 M hydrochloric acid and 50% sodium nitrite for 15 min at room temperature. Degen and Waller used almost the same derivative for measurement but Degen used GC-ECD and Waller used HPLC on C18 with UV detection at 230 nm. The LLOQ by GC-ECD [3] were 5 ng/mL plasma for the sum of free and "apparent" dihydralazine. The detection limit of HPLC-UV [4,5] was 2.5 ng/mL also for the sum. Rouan and Campestrini [1] used similar derivatization steps and HPLC-fluorescence (Ex 230/Em 430 nm) with a detection limit of 1 ng/mL plasma for the sum. Siegmund et al. [2] used almost the same procedure as Degen et al. [3] but with GC-NPD resulting in a detection limit of 5 ng/mL plasma for the sum.

All these methods lack stability of dihydralazine in whole blood and plasma and as our results show the problem of loosing most of the dihydralazine because the loss of free dihydralazine

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cannot be balanced with the "apparent" dihydralazine (only about 10–30%).

2. Experimental, results and discussion of investigations concerning stability and regaining of dihydralazine and HPLC-MS/MS parameters

As described by all authors [1–4] dihydralazine easily forms hydrazones with endogenous aldehydes and ketones. These hydrazones should be easily cleavable and the so regained dihydralazine continues to react via two – very similar for all four papers – steps of derivatization. We worked according to these papers but could release only up to a maximum of 20% of the plasma-bound dihydralazine and either derivatize this part – like published – or detect the free released substance without derivatization with HPLC-tandem-MS. The formed derivates were checked with HPLC-UV, HPLC-fluorescence, HPLC-MS, and HPLC-MS/MS to get a general idea. Derivatization of the analyte worked well with matrix free solutions but with plasma samples yield of only about 5–20% could be achieved. The most critical and all-dominant difficulty was instability of dihydralazine in plasma and even worse in whole blood because the disappeared (free) dihydralazine could hardly be regained (maximum of 5–10% regaining). Data of Rouan and Campestrini [1] in their Fig. 3 regarding free and apparent dihydralazine could not be verified at all, where they got quite good conformity of both values in plasma after 30–60 min at room temperature. We determined a half-life time of free dihydralazine in human

Table 1
Stability of dihydralazine in human plasma at room temperature (with DTT)

Sample	Filename	Calc. conc. (ng/mL)	Mean (ng/mL)	CV (%)	Exp. conc. (ng/mL)	Accuracy (%)	Accuracy ref. mean (%)
10 min							
QC-A1	D3704_C2-029	1.41	1.35	4.90	1.34	105.2	100.7
	D3704_C2-030	1.36			1.34	101.4	
	D3704_C2-031	1.28			1.34	95.4	
QC-B1	D3704_C2-041	18.4	19.0	3.22	18.2	100.8	104.6
	D3704_C2-042	19.5			18.2	107.2	
	D3704_C2-043	19.3			18.2	105.8	
QC-C1	D3704_C2-053	259	254	3.05	258	100.1	98.5
	D3704_C2-054	246			258	95.0	
	D3704_C2-055	259			258	100.4	
20 min							
QC-A1	D3704_C2-061	1.38	1.29	5.97	1.34	102.9	96.4
	D3704_C2-062	1.23			1.34	92.0	
	D3704_C2-063	1.26			1.34	94.3	
QC-B1	D3704_C2-080	16.2	16.3	1.79	18.2	89.1	89.4
	D3704_C2-081	16.6			18.2	91.1	
	D3704_C2-082	16.0			18.2	87.9	
QC-C1	D3704_C2-099	200	202	1.79	258	77.4	78.0
	D3704_C2-100	206			258	79.6	
	D3704_C2-101	199			258	77.0	
40 min							
QC-A1	D3704_C2-067	0.957	0.975	2.58	1.34	71.5	72.8
	D3704_C2-068	0.965			1.34	72.0	
	D3704_C2-069	1.00			1.34	75.0	
QC-B1	D3704_C2-086	14.2	14.1	2.33	18.2	77.9	77.4
	D3704_C2-087	14.4			18.2	78.8	
	D3704_C2-088	13.7			18.2	75.4	
QC-C1	D3704_C2-105	176	181	2.80	258	67.9	70.2
	D3704_C2-106	185			258	71.5	
	D3704_C2-107	184			258	71.2	
60 min							
QC-A1	D3704_C2-073	0.883	0.892	4.25	1.34	65.9	66.6
	D3704_C2-074	0.934			1.34	69.8	
	D3704_C2-075	0.860			1.34	64.2	
QC-B1	D3704_C2-092	12.3	11.8	3.75	18.2	67.6	64.9
	D3704_C2-093	11.7			18.2	64.2	
	D3704_C2-094	11.5			18.2	62.9	
QC-C1	D3704_C2-111	156	153	2.47	258	60.3	59.3
	D3704_C2-112	155			258	59.8	
	D3704_C2-113	149			258	57.6	

plasma of about 10 min at room temperature. Published papers [2–4] give very little information on the gaining of plasma (time, temperature, additives). Rouan and Campestrini [1] mentioned that plasma must be derivatized within 5 min after blood withdrawal at the latest which is almost impossible to perform in such a short time.

2.1. Investigations regarding stability

Thereafter we focussed more on how to stabilize dihydralazine in plasma and whole blood with the entire process of gaining plasma from whole blood.

All results were gained with HPLC-MS/MS determining free dihydralazine.

Since oxidation is the main problem of instability of dihydralazine, addition of some chemically reducing substances like sodium borohydrid, triisobutylphosphine, ascorbic acid and 1,4-dithiothreitol (DTT) were made to plasma.

DTT only showed significant stabilisation effect. A solution for the whole problem could be found in combining DTT and the decrease of temperature immediately after blood withdrawal during gaining of plasma from that.

Tables 1–4 show stability data with/without DTT at 25 °C/0 °C after method validation of dihydralazine in human plasma. Stability in human plasma under best conditions (addition of DTT at ice water temperature of 0 °C) is given for at least 30–40 min. Further investigations were performed by spiking dihydralazine to whole blood with addition of DTT

Table 2
Stability of dihydralazine in human plasma stored at 0 °C (with DTT)

Sample	Filename	Calc. conc. (ng/mL)	Mean (ng/mL)	CV (%)	Exp. conc. (ng/mL)	Accuracy (%)	Accuracy ref. mean (%)
10 min							
QC-A1	D3704_C2-032	1.49	1.48	0.43	1.34	111.0	110.7
	D3704_C2-033	1.96			1.34	[146.4]	
	D3704_C2-034	1.48			1.34	110.4	
QC-B1	D3704_C2-044	20.7	20.9	1.19	18.2	113.7	114.9
	D3704_C2-045	21.2			18.2	116.4	
	D3704_C2-046	20.9			18.2	114.5	
QC-C1	D3704_C2-056	350	301	2.08	258	[135.3]	116.6
	D3704_C2-057	306			258	118.3	
	D3704_C2-058	297			258	114.8	
20 min							
QC-A1	D3704_C2-064	1.32	1.43	7.73	1.34	98.4	106.6
	D3704_C2-065	1.54			1.34	114.9	
	D3704_C2-066	1.43			1.34	106.7	
QC-B1	D3704_C2-083	18.2	18.4	1.07	18.2	99.8	100.9
	D3704_C2-084	18.4			18.2	101.0	
	D3704_C2-085	18.6			18.2	102.0	
QC-C1	D3704_C2-102	240	243	3.27	258	93.1	93.9
	D3704_C2-103	252			258	97.4	
	D3704_C2-104	236			258	91.4	
40 min							
QC-A1	D3704_C2-070	1.32	1.43	6.84	1.34	98.3	106.7
	D3704_C2-071	1.49			1.34	111.1	
	D3704_C2-072	1.48			1.34	110.8	
QC-B1	D3704_C2-089	17.9	18.1	0.82	18.2	98.5	99.4
	D3704_C2-090	18.2			18.2	99.8	
	D3704_C2-091	18.2			18.2	99.9	
QC-C1	D3704_C2-108	235	240	2.25	258	90.8	92.7
	D3704_C2-109	245			258	95.0	
	D3704_C2-110	239			258	92.3	
60 min							
QC-A1	D3704_C2-076	1.30	1.39	7.06	1.34	97.1	104.1
	D3704_C2-077	1.39			1.34	103.5	
	D3704_C2-078	1.50			1.34	111.7	
QC-B1	D3704_C2-095	16.6	17.1	2.55	18.2	91.1	93.8
	D3704_C2-096	17.2			18.2	94.4	
	D3704_C2-097	17.4			18.2	95.8	
QC-C1	D3704_C2-114	231	228	1.48	258	89.5	88.1
	D3704_C2-115	225			258	87.0	
	D3704_C2-116	227			258	87.9	

Table 3
Stability of dihydralazine in human plasma without DTT at room temperature

Sample	Filename	Calc. conc. (ng/mL)	Mean (ng/mL)	CV (%)	Exp. conc. (ng/mL)	Accuracy (%)	Accuracy ref. mean (%)
Start							
QC-A2	D3704_C4-032	0.912	0.890	3.46	1.34	68.1	66.4
	D3704_C4-033	0.855			1.34	63.8	
	D3704_C4-034	0.903			1.34	67.4	
QC-B2	D3704_C4-048	15.5	15.1	3.04	18.2	85.0	82.7
	D3704_C4-049	14.6			18.2	80.0	
	D3704_C4-050	15.1			18.2	83.0	
QC-C2	D3704_C4-064	225	211	5.75	258	87.1	81.8
	D3704_C4-065	206			258	79.9	
	D3704_C4-066	202			258	78.3	
10 min							
QC-A2	D3704_C4-035	<LLOQ	n/a	n/a	1.34	n/a	n/a
	D3704_C4-036	<LLOQ			1.34	n/a	
	D3704_C4-037	<LLOQ			1.34	n/a	
QC-B2	D3704_C4-051	7.34	7.39	10.73	18.2	40.3	40.6
	D3704_C4-052	8.21			18.2	45.1	
	D3704_C4-053	6.63			18.2	36.4	
QC-C2	D3704_C4-067	138	138	2.53	258	53.3	53.5
	D3704_C4-068	142			258	54.9	
	D3704_C4-069	135			258	52.3	
20 min							
QC-A2	D3704_C4-041	<LLOQ	n/a	n/a	1.34	n/a	n/a
	D3704_C4-042	<LLOQ			1.34	n/a	
	D3704_C4-043	<LLOQ			1.34	n/a	
QC-B2	D3704_C4-057	3.59	3.58	1.15	18.2	19.7	19.7
	D3704_C4-058	3.61			18.2	19.8	
	D3704_C4-059	3.53			18.2	19.4	
QC-C2	D3704_C4-073	97.2	96.7	1.08	258	37.6	37.4
	D3704_C4-074	97.4			258	37.7	
	D3704_C4-075	95.5			258	37.0	
40 min							
QC-A2	D3704_C4-106	<LLOQ	n/a	n/a	1.34	n/a	n/a
	D3704_C4-107	<LLOQ			1.34	n/a	
	D3704_C4-108	<LLOQ			1.34	n/a	
QC-B2	D3704_C4-093	1.41	1.38	3.48	18.2	7.7	7.6
	D3704_C4-094	1.33			18.2	7.3	
	D3704_C4-095	1.41			18.2	7.7	
QC-C2	D3704_C4-080	51.8	50.0	5.47	258	20.1	19.4
	D3704_C4-081	51.3			258	19.9	
	D3704_C4-082	46.9			258	18.1	
60 min							
QC-A2	D3704_C4-112	<LLOQ	n/a	n/a	1.34	n/a	n/a
	D3704_C4-113	<LLOQ			1.34	n/a	
	D3704_C4-114	<LLOQ			1.34	n/a	
QC-B2	D3704_C4-099	1.11	1.13	6.58	18.2	6.1	6.2
	D3704_C4-100	1.21			18.2	6.6	
	D3704_C4-101	1.07			18.2	5.9	
QC-C2	D3704_C4-086	36.1	32.6	9.36	258	14.0	12.6
	D3704_C4-087	31.4			258	12.2	
	D3704_C4-088	30.3			258	11.7	

at ice water temperature which led to a safe blood sampling procedure (with regard to stability; no significant loss of the analyte during the whole sample gaining/sample preparation process).

Stability over 1 month is given when storing frozen plasma samples (with DTT) at −60 °C. Since stability in human plasma (after adding DTT) at room temperature is given for 10 min only, just six samples were thawed at a time and

Table 4
Stability of dihydralazine in human plasma stored at 0 °C without DTT

Sample	Filename	Calc. conc. (ng/mL)	Mean (ng/mL)	CV (%)	Exp. conc. (ng/mL)	Accuracy (%)	Accuracy ref. mean (%)
Start							
QC-A2	D3704_C4-032	0.912	0.890	3.46	1.34	68.1	66.4
	D3704_C4-033	0.855			1.34	63.8	
	D3704_C4-034	0.903			1.34	67.4	
QC-B2	D3704_C4-048	15.5	15.1	3.04	18.2	85.0	82.7
	D3704_C4-049	14.6			18.2	80.0	
	D3704_C4-050	15.1			18.2	83.0	
QC-C2	D3704_C4-064	225	211	5.75	258	87.1	81.8
	D3704_C4-065	206			258	79.9	
	D3704_C4-066	202			258	78.3	
10 min							
QC-A2	D3704_C4-038	0.539	0.546	1.88	1.34	40.3	40.8
	D3704_C4-039	<LLOQ			1.34	n/a	
	D3704_C4-040	0.554			1.34	41.3	
QC-B2	D3704_C4-054	9.66	10.5	7.67	18.2	53.0	57.9
	D3704_C4-055	10.7			18.2	58.8	
	D3704_C4-056	11.3			18.2	61.8	
QC-C2	D3704_C4-070	175	172	1.79	258	67.6	66.7
	D3704_C4-071	169			258	65.3	
	D3704_C4-072	173			258	67.1	
20 min							
QC-A2	D3704_C4-044	<LLOQ	n/a	n/a	1.34	n/a	n/a
	D3704_C4-045	<LLOQ			1.34	n/a	
	D3704_C4-046	<LLOQ			1.34	n/a	
QC-B2	D3704_C4-060	8.56	8.95	4.61	18.2	47.0	49.1
	D3704_C4-061	9.38			18.2	51.5	
	D3704_C4-062	8.89			18.2	48.8	
QC-C2	D3704_C4-076	161	158	4.77	258	62.4	61.0
	D3704_C4-077	163			258	63.0	
	D3704_C4-078	149			258	57.7	
40 min							
QC-A2	D3704_C4-109	<LLOQ	n/a	n/a	1.34	n/a	n/a
	D3704_C4-110	<LLOQ			1.34	n/a	
	D3704_C4-111	<LLOQ			1.34	n/a	
QC-B2	D3704_C4-096	4.81	4.77	11.01	18.2	26.4	26.2
	D3704_C4-097	5.28			18.2	29.0	
	D3704_C4-098	4.23			18.2	23.2	
QC-C2	D3704_C4-083	106	110	5.30	258	41.0	42.6
	D3704_C4-084	108			258	41.6	
	D3704_C4-085	117			258	45.2	
60 min							
QC-A2	D3704_C4-115	<LLOQ	n/a	n/a	1.34	n/a	n/a
	D3704_C4-116	<LLOQ			1.34	n/a	
	D3704_C4-117	<LLOQ			1.34	n/a	
QC-B2	D3704_C4-102	2.81	2.89	3.39	18.2	15.4	15.9
	D3704_C4-103	3.00			18.2	16.5	
	D3704_C4-104	2.87			18.2	15.7	
QC-C2	D3704_C4-089	82.4	84.7	3.03	258	31.9	32.8
	D3704_C4-090	87.5			258	33.9	
	D3704_C4-091	84.2			258	32.6	

precipitated with trichloroacetic acid (TCA). After precipitation of human plasma proteins dihydralazine is stable in supernatant for at least 18 h at 5 °C (cooled auto sampler tray).

2.2. Investigations regarding HPLC-tandem-MS

As dihydralazine is a very reactive molecule, some difficulties with direct measurement arose regarding tailing of the peak and

carryover. Peak shape was bad although we tested several different reversed phase materials (even with special endcapping) until – after using some ion pairing reagents – we ended up using trichloroacetic acid (TCA). TCA provided hardly any tailing and good linearity of the MS signal with increasing concentration but carryover remained high at about 3%. Thereafter we added some TCA to the injector flush solution (50% acetonitrile in water) which reduced carryover a little bit. Acceptable carryover values (should be lower than 0.1%) for the method were still out of reach so various hydrazines in very low concentrations were added to the mobile phase. Finally 20 mg isonicotinic hydrazine/L of mobile phase gave best results. Looking for the most appropriate internal standard (neither deuterated nor isotopically labelled dihydralazine available) was difficult. After some testing in plasma samples, Metoprolol was chosen as internal standard (IS). Substances with medium or high protein binding could not be taken as internal standard since TCA does not cleave protein binding and therefore individual plasma samples could have different protein binding and consequently different internal standard recovery.

3. Experimental (validated method)

3.1. Chemicals, reference items and matrices

Chemicals used were acetonitrile (gradient grade), methanol (gradient grade), hydrochloric acid (p.A., 25% in water), and trichloroacetic acid (p.A., 20% in water) by Merck, Germany. Furthermore 1,4-dithiothreitol (p.A.) provided by Roche, Switzerland; isonicotinic hydrazine (p.A.) provided by Fluka, Switzerland, and water (ASTM-1 grade) by pharm-analyt, Austria.

Reference item was dihydralazine provided by Medinex Laboratories, India and the internal standard Metoprolol (IS) provided by Sigma–Aldrich, USA.

Heparinised human plasma used for spiking of Calibration Standards and Quality Control Samples was pooled by pharm-analyt, Austria. Study samples resulted from a single dose (25 mg) crossover bioavailability study with 24 healthy volunteers.

3.2. Sample preparation

To comply stability times of dihydralazine not more than six samples were prepared at the same time. All plasma samples (Calibration Standards, Quality Control Samples, other Control Samples, study samples at the clinical site after blood withdrawal) were added 1% (v/v) of a 5% DTT solution (w/v in water).

Previous to the transfer of 200 μL of the study samples, 50 μL of internal standard working solution and 50 μL of 20% TCA (w/v) were put together in each sample vial (volume approx. 5 mL). After thawing at approximately 20–25 °C in a water bath for a maximum of 5 min the samples were prepared for analysis. In the first step 200 μL of the study samples were transferred into the sample vials prepared before. Immediately thereafter the vials were vortexed for approx. 20 s. Subsequently the vials were centrifuged at 3040 × *g* for 2 min. Then 150 μL of the clear supernatant was transferred into conical auto sampler vials. These vials were sealed with an aluminium crimp cap and used for HPLC within 18 h (auto sampler temperature +5 °C).

3.3. High performance liquid chromatography

Pumps	PE Series 200 Micro Pump (Perkin-Elmer, USA)
Auto sampler	PE Series 200 Auto sampler (Perkin-Elmer, USA)
Column oven	Jetstream 2 Plus (W. O. Electronics, Austria)
Mobile phase	Solvent A: 20 mM TCA in water (containing 20 mg isonicotinic hydrazine/L) Solvent B: 20 mM TCA in acetonitrile (containing 20 mg isonicotinic hydrazine/L)
Gradient	0.0–0.1 min isocratic: 0% B 0.1–1.5 min linear: 0% B → 20% B 1.5–4.1 min linear: 20% B → 75% B 4.1–5.0 min isocratic: 0% B
Column	Synergi polar RP 80 A, 100 mm × 2 mm, 4 μm (Phenomenex, USA)
Flow	0.6 mL/min
Temperature	40 °C
Injection volume	20 μL
Retention time	Approx. 2.1 min: dihydralazine Approx. 3.2 min: IS
Sample storage in auto sampler	5 °C (cooled Peltier tray)

3.4. Mass spectrometer parameters

Mass spectrometer	API 3000 (PE Sciex, Canada)
Operational mode	Turbo Spray (ESI), in positive ion mode
Vaporizer temperature	500 ± 10 °C
Ion spray voltage	5000 V
Nebulizer gas	Flow = 10 device units; pressure = 4 bar
Curtain gas	Flow = 15 device units
Horizontal position	Approx. 15 mm
Lateral position	Approx. 5 mm (to the left)
Quadrupol resolution	Low → low
Detection mode	MRM
Transitions	191.3 → 129.1 *m/z*: dihydralazine 268.3 → 116.1 *m/z*: IS

3.5. Method validation

The analytical method was validated in three batches (including demonstration of linearity, accuracy, precision, specificity, recovery and LLOQ). Minimum one set of Calibration Standards and five sets of Quality Control Samples were analysed within these three different batches as well as a carryover, a blank, and a zero sample. Calibration Standards were made at eight concentration levels by adding defined volumes of aqueous solutions containing dihydralazine or a higher concentrated Calibration Standard to analyte-free human plasma. Quality Control Samples were prepared alike but spiked in a different batch of human plasma with different solutions of the analyte deriving from a second weighing. Concentrations of Calibration Standards were

between 0.500 ng/mL and 302 ng/mL of the analyte in human plasma and at 1.34/18.2/258 ng/mL for Quality Control Samples.

To determine the assay's linearity, precisions (coefficient of variations, CVs) and accuracies at least three batches should be analysed, each consisting of at least one set of Calibration Standards, a zero sample (or Standard 0), a blank sample and five QC-samples at each of three concentration levels.

Intra- (for QC-samples only) and inter-batch (for Calibration Standards and QC-samples) precisions (as CVs in %) and accuracies (in %) of the assay should be derived from the results of the validation batches mentioned above.

Concentration values of both the Calibration Standards and the QC-samples should be back-calculated from the appropriate calibration curve. Thereof inter-batch mean values, precisions (as CVs) and accuracies should be calculated.

3.6. Specificity

Acceptable specificity was defined as an area of possible interferences in human plasma in blank and zero samples. "Blank sample" refers to Standard 0 without analyte and internal standard and "zero sample" refers to Standard 0 with internal standard. Blank and zero samples had to be below 1/3 of the area of Calibration Standard 1 (at level of LLOQ) or not detectable. The specificity of the method was determined by analysing six sample pairs consisting of one zero and one blank human plasma sample per volunteer.

3.7. Recovery

Recovery was determined by comparing the areas of Quality Control Samples with areas of aqueous solutions (without sample preparation but appropriate dilution in accordance to sample preparation of QC-samples) at three concentration levels. Each peak area of the QC-samples was divided by the mean peak area of those direct aqueous solutions. Aqueous DIR-samples had to be analysed in triplicate at each of these concentration levels.

3.8. Method linearity

The calibration range was from 0.500 ng/mL to 302 ng/mL for the analyte in human plasma. The inter-batch coefficient of variation had to be <15% (20% at LLOQ level) for precision, and for accuracy the mean value had to be within ±15% of the actual value (20% at LLOQ level). However, at LLOQ level, 20% was acceptable for both inter-batch precision and accuracy. If the calibration curve was rejected, the batch had to be rejected also. A quadratic regression with a weighting factor $1/x^2$ should be used (after pre-study tests). The coefficient of correlation (R) has to achieve a degree of certainty of $R = 0.99$.

Accuracy of individual calculated values must not deviate more than ±15% (20% at LLOQ level) from their expected ones.

Seventy-five percent of all individual values, but at least six concentration levels have to match the specifications mentioned above.

If any values failed (⟨oos⟩), the respective calibration curve will be calculated anew without them, retaining the upper conditions unchanged. At least 50% of all individual values at a certain concentration level have to be valid, else this concentration level fails (⟨oos⟩).

⟨oos⟩ concentration levels are allowed unless those being adjacent ones.

3.9. Precision and accuracy for QC-samples

Five replicates of Quality Control Samples at three concentration levels each had to be analysed. Quality Control Samples were prepared at three concentrations (≤3x LLOQ, mid-range and at least 80% of the highest calibration concentration) and were, at least in triplicate, incorporated into each sequence. According to the results of the QC-samples, a sequence was accepted or rejected. At least six of the nine QC-samples had to be within ±15% of their respective nominal values; three of the nine QC-samples (but not at the same concentration) may have been outside the ±15% of their respective nominal values.

If a batch did not adhere to these criteria, the batch was rejected. QC-samples outside ±15% (±20% at LLOQ level) are called "out of specifications", whereas QC-samples outside ±30% (±40% at LLOQ level) are called "outlier".

4. Results and discussion

After clearing of all the problems in regards to instability in human plasma and chromatography (tailing, carryover) a method with good precision and accuracy resulted. Figs. 1 and 2 display chromatograms of blank plasma without IS (Fig. 1) and with IS (Fig. 2). Fig. 3 shows a chromatogram at LLOQ level and Fig. 4 at the highest concentration.

The stability of dihydralazine in plasma with/without DTT at 0 °C (ice water) and room temperature (about 25 °C) is shown in Fig. 5 for QC-A level (1.34 ng/mL), for QC-B level (18.2 ng/mL) in Fig. 6 and for QC-C level (258 ng/mL) in Fig. 7.

Table 5
Regression parameters and carryover of dihydralazine in human plasma

Sequence	a	b	c	R	Cal. range (ng/mL)	n of stds.	w.F.	Carry-over (%)
DHL_3704_C1	−2.07E−6	0.00276	3.45E−5	0.9952	0.500–302	9	$1/x^2$	0.01
DHL_3704_C2	−1.28E−6	0.00257	0.000227	0.9995	0.500–302	9	$1/x^2$	0.01
DHL_3704_C3	−1.71E−6	0.00254	0.000204	0.9994	0.500–302	9	$1/x^2$	0.01
DHL_3704_C4	−1.25E−6	0.00217	2.09E−5	0.9984	0.500–302	9	$1/x^2$	0.05

$y = ax^2 + bx + c$

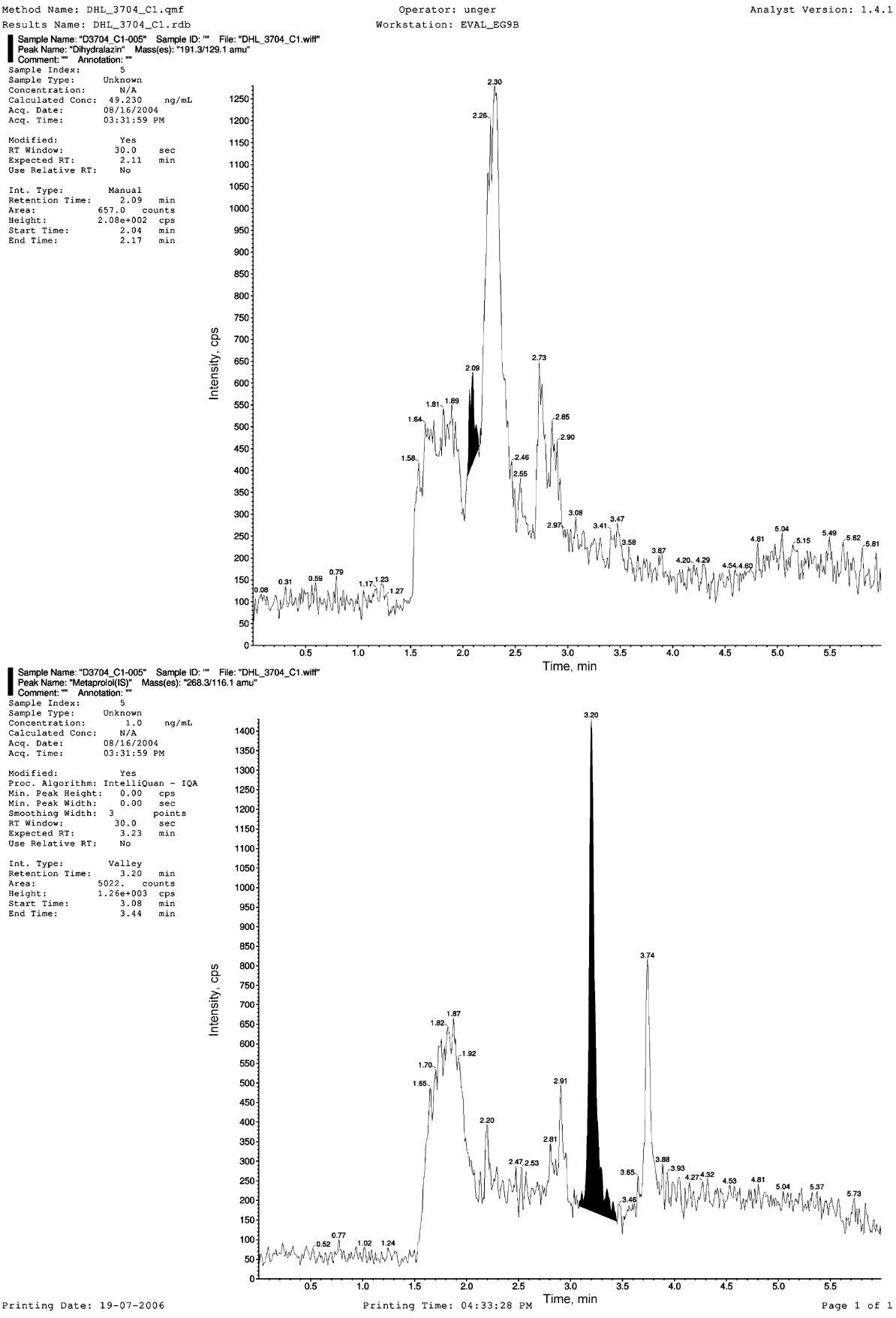


Fig. 1. Chromatogram of blank sample (without IS and any analyte).

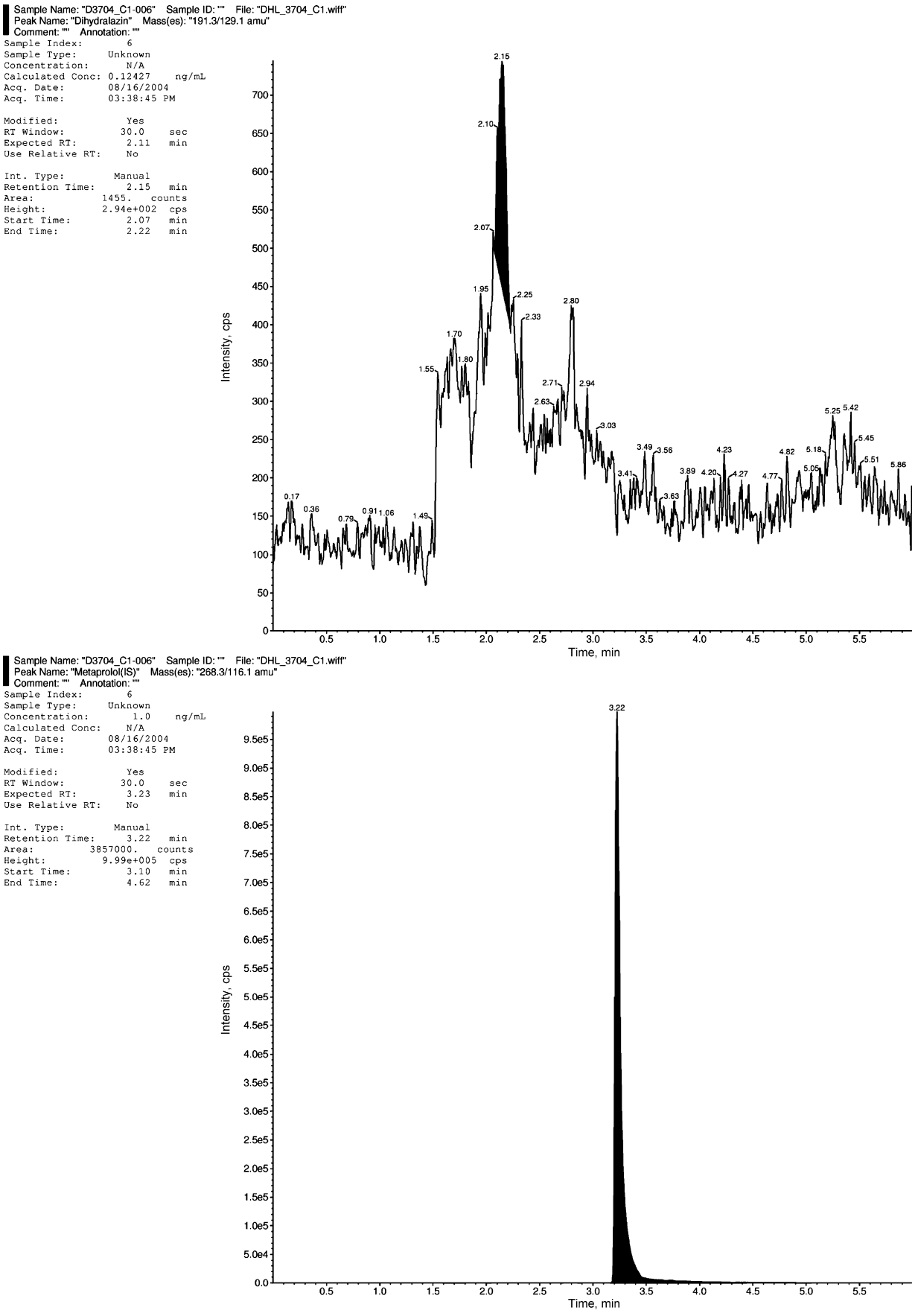


Fig. 2. Chromatogram of zero sample.

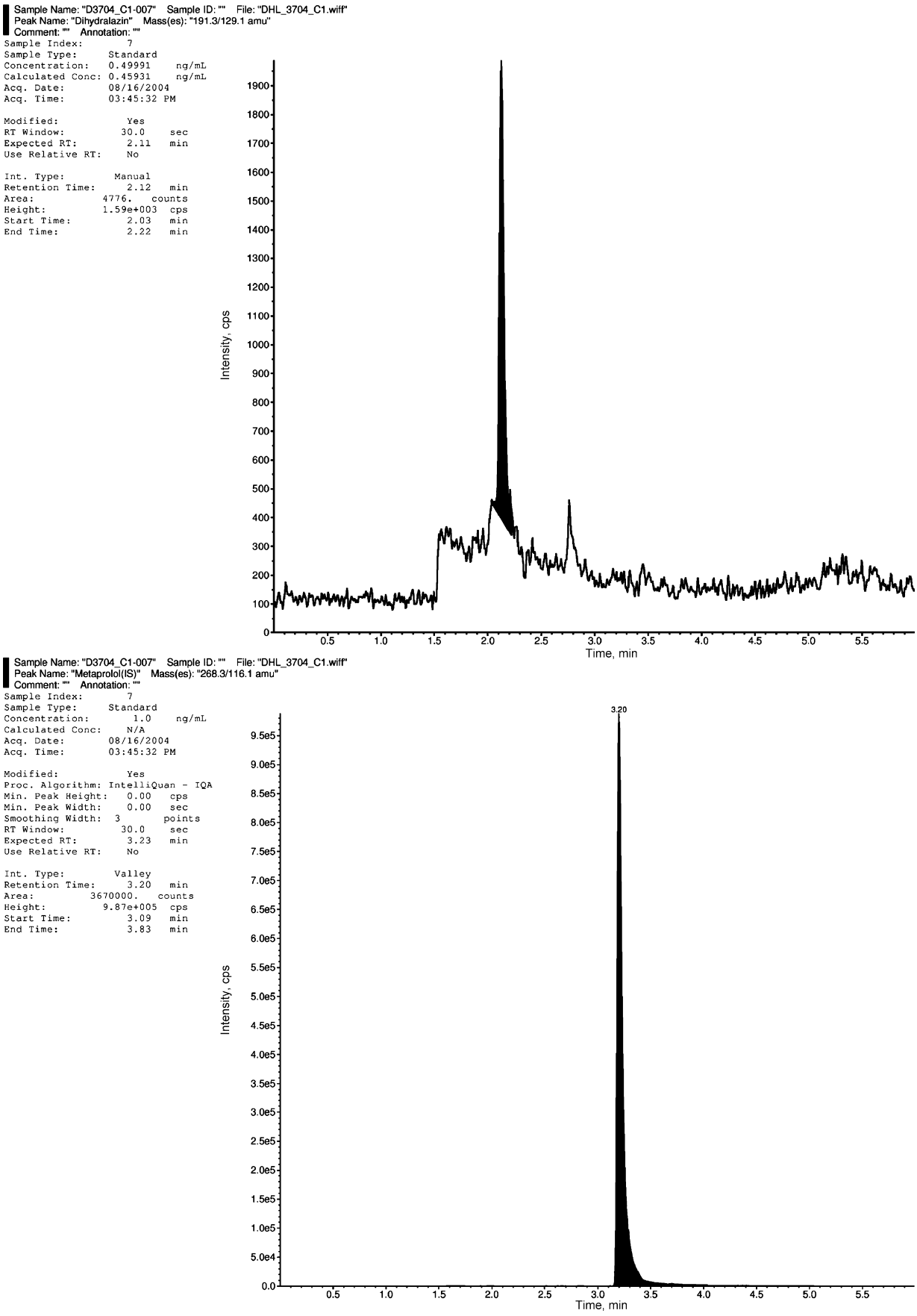
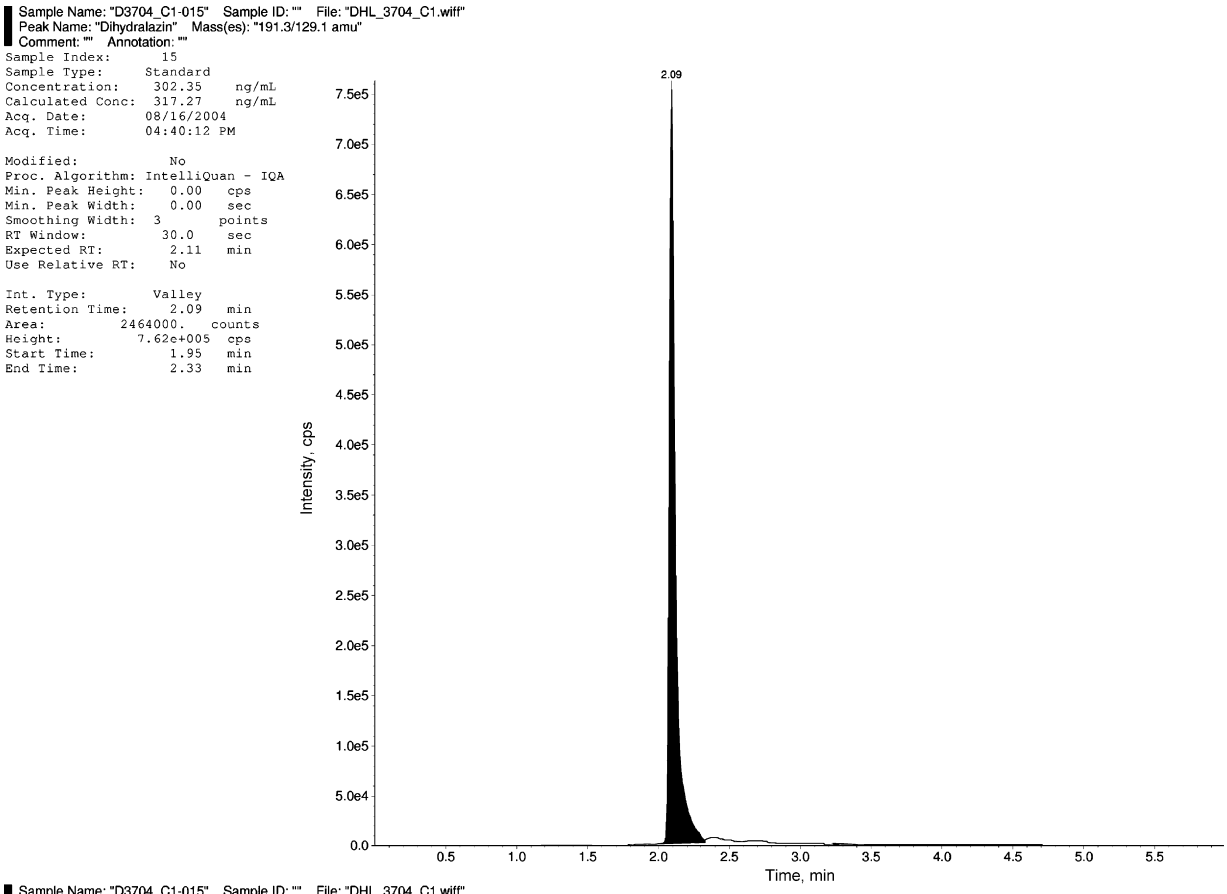


Fig. 3. Chromatogram of Calibration Standard Std1A (at level of LLOQ).



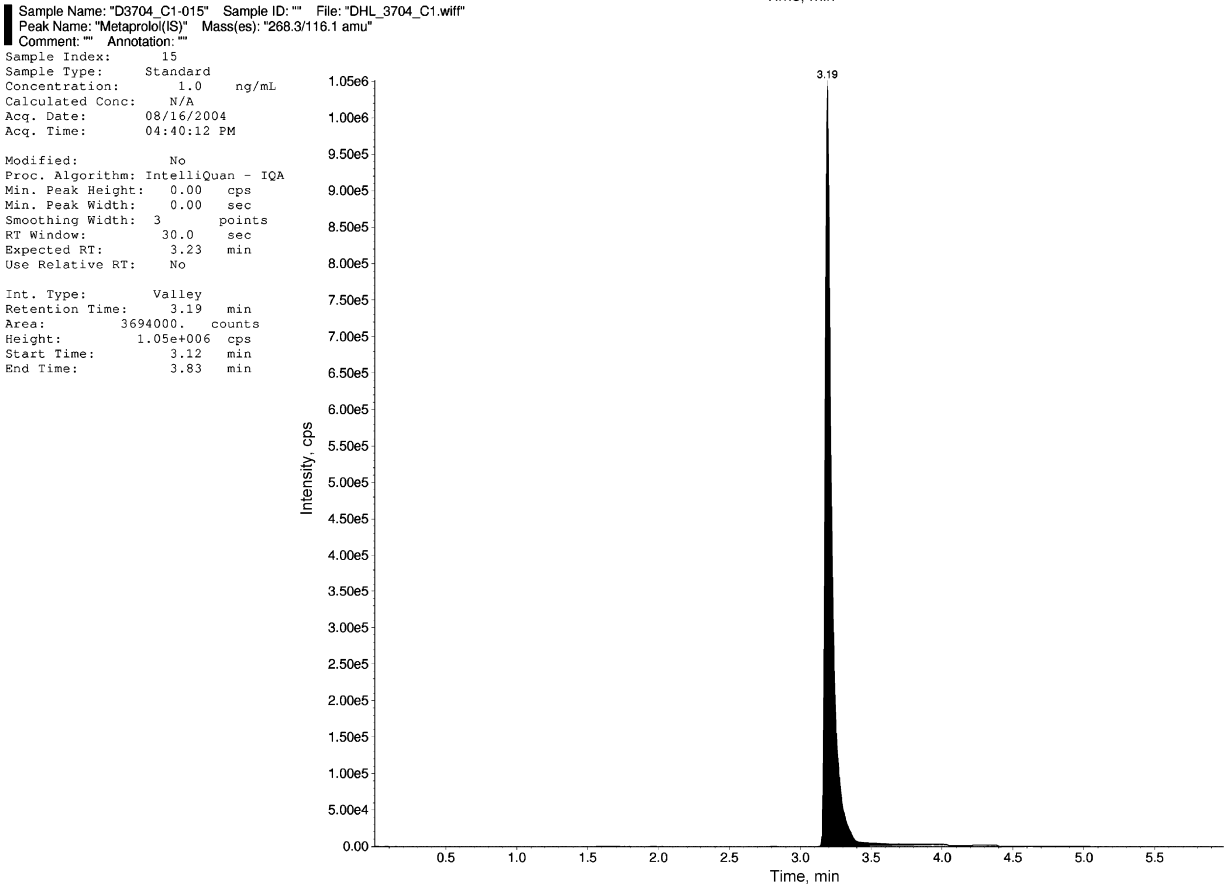


Fig. 4. Chromatogram of Calibration Standard Std9A (at level of ULOQ).

Table 6
Linearity, precision (CV) and accuracy of dihydralazine in human plasma

Sequence	Unit	Calculated concentration (ng/mL)								
		Std1A	Std2A	Std3A	Std4A	Std5A	Std6A	Std7A	Std8A	Std9A
DHL_3704_C1	ng/mL	0.459	1.15	2.89	6.18	17.0	44.3	92.1	209	317
DHL_3704_C2	ng/mL	0.501	1.03	2.54	6.31	17.8	46.9	103	216	288
DHL_3704_C3	ng/mL	0.490	1.05	2.67	6.31	17.7	47.0	97.2	203	311
DHL_3704_C4	ng/mL	0.490	1.03	2.83	6.40	17.1	44.5	101	201	311
Mean	ng/mL	0.485	1.06	2.73	6.30	17.4	45.7	98.4	207	307
S.D.	ng/mL	0.018	0.06	0.16	0.09	0.4	1.5	4.9	7	13
CV	%	3.70	5.75	5.69	1.45	2.32	3.18	4.98	3.34	4.26
Number		4	4	4	4	4	4	4	4	4
Exp. conc.	ng/mL	0.500	1.03	2.58	6.19	18.6	46.2	102	205	302
Accuracy	%	97.1	103.8	105.9	101.8	93.4	99.0	96.6	101.4	101.5

Table 7
Intra- and inter-batch precision (CV) and accuracy of dihydralazine (QC-A1)

Quality Control Sample QC-A1 (expected concentration 1.34 ng/mL)

Sequence	Calculated concentration (g/mL)					Mean (ng/mL)	CV (%)	Accuracy (%)
	Value 1	Value 2	Value 3	Value 4	Value 5			
DHL_3704_C1	1.34	1.43	1.42	1.51	1.46	1.43	4.22	106.9
DHL_3704_C2	1.54	1.45	1.44	1.49	1.53	1.49	3.12	111.2
DHL_3704_C3	1.32	1.32	1.32	1.28	1.17	1.28	4.92	95.6
DHL_3704_C4	1.37	1.43	1.22	1.39	1.23	1.33	7.32	99.2

Acceptable range (85–115%): 1.14–1.54 ng/mL

Inter-batch precision and accuracy (reproducibility), $n = 4$ batches

Mean (ng/mL)	1.38
S.D. (ng/mL)	0.11
CV (%)	7.66
Number	20
Exp. conc. (ng/mL)	1.34
Accuracy (%)	103.2

The overall recovery for dihydralazine was determined to be 103.8%. The mean recovery for the internal standard was determined to be 111.0%.

Aliquots of a sample spiked to the five-fold analyte concentration of the level of the highest QC-sample were diluted (v/v = 1/10) with analyte-free matrix prior to the sample preparation for analysis. The calculated results of these "dilution samples" met the criteria in respect of CV and accuracy.

The linearity and carryover is presented in Table 5. Linearity, precision and accuracy of Calibration Standards are shown in Table 6. The intra- and inter-batch precision and accuracies of the QC-samples are presented in Tables 7–9. The stability in injection solution at 5 °C over 10 and 18 h and after one freeze/thaw cycle is presented in Table 10. The results of LLOQ samples (inter-batch precision and accuracy) are shown in Table 11. Using this method study samples from a single dose (25 mg) crossover bioavailability study with 24 healthy volunteers were analysed. Fig. 8 shows a representative pharmacokinetic profile after oral administration of 25 mg of dihydralazine to a volunteer.

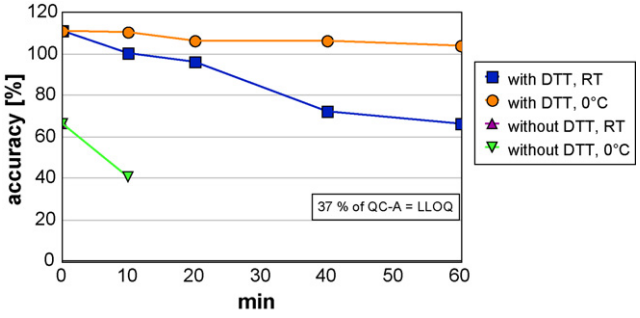


Fig. 5. Stability of dihydralazine in human plasma (concentration level QC-A).

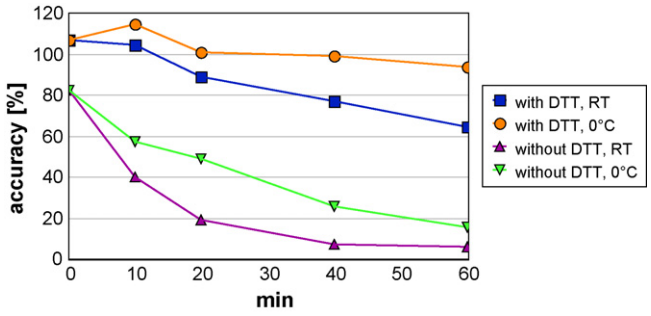


Fig. 6. Stability of dihydralazine in human plasma (concentration level QC-B).

Table 8
Intra- and inter-batch precision (CV) and accuracy of dihydralazine (QC-B1)

Quality Control Sample QC-B1 (expected concentration 18.2 ng/mL)								
Sequence	Calculated concentration (ng/mL)					Mean (ng/mL)	CV (%)	Accuracy (%)
	Value 1	Value 2	Value 3	Value 4	Value 5			
DHL_3704_C1	17.4	17.9	18.4	19.4	18.5	18.3	4.05	100.5
DHL_3704_C2	[25.5]	20.9	20.4	19.6	17.3	19.5	8.21	107.3
DHL_3704_C3	18.3	17.4	17.0	17.8	16.6	17.4	3.91	95.6
DHL_3704_C4	19.2	19.0	21.6	17.5	16.4	18.7	10.50	102.8

Acceptable range (85–115%): 15.5–20.9 ng/mL

Inter-batch precision and accuracy (reproducibility), $n = 4$ batches	
Mean (ng/mL)	18.4
S.D. (ng/mL)	1.5
CV (%)	7.86
Number	19
Exp. conc. (ng/mL)	18.2
Accuracy (%)	101.3

Table 9
Intra- and inter-batch precision (CV) and accuracy of dihydralazine (QC-C1)

Quality Control Sample QC-C1 (expected concentration 258 ng/mL)								
Sequence	Calculated concentration (ng/mL)					Mean (ng/mL)	CV (%)	Accuracy (%)
	Value 1	Value 2	Value 3	Value 4	Value 5			
DHL_3704_C1	275	237	249	250	251	252	5.46	97.6
DHL_3704_C2	294	284	283	252	236	270	9.11	104.4
DHL_3704_C3	244	235	239	221	204	229	7.09	88.6
DHL_3704_C4	266	268	287	281	225	265	9.14	102.7

Acceptable range (85–115%): 220–297 ng/mL

Inter-batch precision and accuracy (reproducibility), $n = 4$ batches	
Mean (ng/mL)	254
S.D. (ng/mL)	25
CV (%)	9.73
Number	20
Exp. conc. (ng/mL)	258
Accuracy (%)	98.3

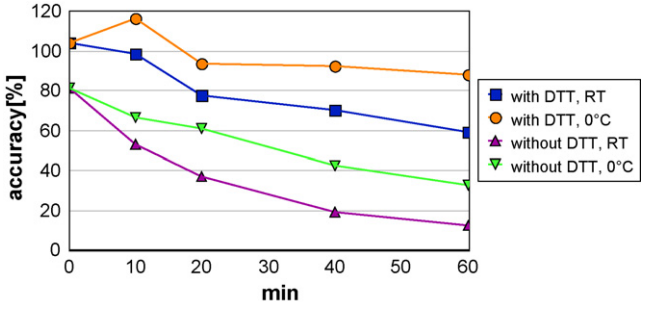


Fig. 7. Stability of dihydralazine in human plasma (concentration level QC-C).

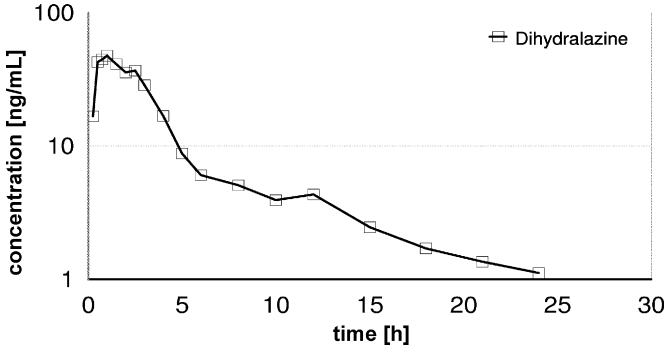


Fig. 8. Dihydralazine plasma levels after oral application of 25 mg to one volunteer.

Table 10
Stability of dihydralazine after sample preparation

Sample	Filename	Calc. conc. (ng/mL)	Mean (ng/mL)	CV (%)	Exp. conc. (ng/mL)	Accuracy (%)	Accuracy ref. mean (%)
18 h							
QC-A1	D3704_C2-026	1.15	1.22	9.99	1.34	85.6	91.4
	D3704_C2-027	1.16			1.34	86.7	
	D3704_C2-028	1.37			1.34	102.0	
QC-B1	D3704_C2-038	18.1	17.0	5.49	18.2	99.3	93.5
	D3704_C2-039	16.3			18.2	89.5	
	D3704_C2-040	16.7			18.2	91.7	
QC-C1	D3704_C2-050	240	241	2.46	258	92.8	93.3
	D3704_C2-051	236			258	91.3	
	D3704_C2-052	248			258	95.8	
10 h							
QC-A1	D3704_C2-118	1.46	1.36	6.85	1.34	109.2	101.4
	D3704_C2-119	1.28			1.34	95.8	
	D3704_C2-120	1.33			1.34	99.4	
QC-B1	D3704_C2-122	19.0	18.1	5.46	18.2	104.6	99.6
	D3704_C2-123	18.3			18.2	100.4	
	D3704_C2-124	17.1			18.2	93.8	
QC-C1	D3704_C2-126	220	231	4.20	258	85.1	89.4
	D3704_C2-127	235			258	90.8	
	D3704_C2-128	238			258	92.3	
One freeze–thaw cycle							
QC-A1	D3704_C3-081	1.38	1.28	7.04	1.34	103.0	95.6
	D3704_C3-082	1.21			1.34	90.0	
	D3704_C3-083	1.26			1.34	93.7	
QC-B1	D3704_C3-077	15.6	16.5	6.61	18.2	85.9	90.5
	D3704_C3-078	17.7			18.2	97.3	
	D3704_C3-079	16.1			18.2	88.4	
QC-C1	D3704_C3-073	224	223	3.13	258	86.6	86.3
	D3704_C3-074	216			258	83.5	
	D3704_C3-075	230			258	88.9	

Table 11
Lower limit of quantitation of dihydralazine in human plasma

LLOQ (expected concentration 0.500 ng/mL)

Sequence	Calculated concentration (ng/mL)						Mean (ng/mL)	CV (%)	Accuracy (%)
	Value 1	Value 2	Value 3	Value 4	Value 5	Value 6			
DHL_3704_C1	0.555	0.546	0.543	0.560	[0.721]	0.568	0.554	1.86	110.9
DHL_3704_C2	[0.705]	0.597	0.570	0.539	0.473	0.484	0.533	10.09	106.5
DHL_3704_C3	0.466	0.521	0.506	0.560	0.496	0.526	0.513	6.18	102.5

Acceptable range (80–120%): 0.400–0.600 ng/mL

Inter-batch precision and accuracy (reproducibility), $n = 4$ batches

Mean (ng/mL)	0.532
S.D. (ng/mL)	0.038
CV (%)	7.17
Number	16
Exp. conc. (ng/mL)	0.500
Accuracy (%)	106.4

5. Conclusion

HPLC-MS/MS has not been used before in a published paper and provides better sensitivity and selectivity. Therefore a much easier sample preparation than published before is feasible (protein precipitation).

With the described method the determination of dihydralazine in human plasma is possible for the first time described in literature in a reproducible manner. All instability (in plasma and whole blood) and the chromatographic problems (tailing, carryover) could be resolved. Several hundred plasma samples from a single dose study have been analysed with this method.

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