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Improved determination of sulfadiazine in human plasma and urine by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the determination of sulfadiazine in human plasma and human urine was developed and validated. The method involves the acid extraction of drug and internal standard from plasma with ethyl acetate followed by evaporation and reconstitution in mobile phase. Urine samples were simply diluted with purified water. Recovery, linearity, intra- and inter-day variation of sulfadiazine were tested and found appropriate. The quantitation range was 0.0299–15.2 μg/ml for plasma samples and 0.578–148.8 μg/ml for urine samples. The method is suitable for the quantitation of sulfadiazine from pharmacokinetic studies.

Keywords: Sulfadiazine; Sulfamethazine

1. Introduction

In 1981 we developed and published a HPLC method for the determination of sulfadiazine in plasma [1]. However, this method did not include an internal standard and was not very sensitive as the quantitation limit was set to 100 ng/ml. Moreover, the awareness for the need to validate analytical methods has profoundly grown since then. In need for an HPLC method to assay samples from a pharmacokinetic study with Urospasmon, an antibacterial drug containing sulfadiazine and nitrofurantoin, we had to reconsider our methodology. Screening of the literature revealed a number of methods published for the determination of sulfadiazine. Part of those are reporting methodologies for the assay of sulfadiazine in animal tissues [2–4]. Other reported methods do not reach sufficient sensitivity with detection limits of 0.1 μg/ml [5], 1 μg/ml [6] or 2 μg/ml [7] in plasma. Therefore we decided to develop a new method based on our previous method with the aim to include an internal standard and to improve the quantitation limit considerably. The method was extensively validated fulfilling international guidelines [8] and also adapted for measurement of sulfadiazine in urine.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents used were of HPLC grade or analytical grade. Sulfadiazine was supplied by Heumann Pharma (Nürnberg, Germany). Sulfamethazine was purchased from Sigma (St. Louis,

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MO, USA) as the sodium salt. Sodium dihydrogen phosphate monohydrate as well as *o*-phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). Solvents were of analytical and HPLC grade. Acetonitrile and ethyl acetate were of ChromAR quality (Promochem, Wesel, Germany). Water purified by Milli-Q system (Millipore, Bedford, MA, USA) was used in all procedures involving water.

2.2. Apparatus and chromatographic conditions

The HPLC apparatus consisted of a Kontron 420 pump (Kontron Instruments, Neufahrn, Germany), a Shimadzu SDP-6A (Shimadzu, Tokyo, Japan) variable-wavelength UV detector (set at 270 nm) and a 710 B WISP autosampler (Waters, Eschborn, Germany). Integration was performed using the software Turbochrom 3 (Perkin Elmer Nelson, Cupertino, CA, USA) installed on an IBM compatible PC connected to a Star LC-10 printer. The analytical column (stainless steel, 250×4.6 mm I.D.) was packed with 5-μm particles of Spherisorb ODS II (M. Grom, Herrenberg, Germany). Refilled columns were used throughout the validation procedures. As a guard column we used cartridges manufactured by Merck, filled with Lichrospher RP 18, 5 μm (4×4 mm). Guard columns were replaced on a routine system after 60–90 injections (e.g. after one run) during the validation procedures. For analysis of plasma and urine samples the composition of the mobile phase was 20 m*M* NaH_2PO_4 buffer and acetonitrile (93:7, v/v). The pH was adjusted to 4.7 with orthophosphoric acid. The flow-rate of the mobile phase was 1.8 ml/min.

2.3. Preparation of stock solutions, calibration levels and validation samples

A stock solution of 1.5 mg/ml sulfadiazine was prepared in purified water. A stock solution of the internal standard sulfamethazine was prepared by dissolving an appropriate amount of sulfamethazine in purified water to achieve a solution of 50 μg/ml sulfamethazine sodium. Calibration levels in the appropriate drug-free matrix were prepared yielding a concentration range from 0.0299 μg/ml up to 15.2 μg/ml. Validation samples (VS) at three different concentration levels were prepared leading to concentrations of 10.9 μg/ml, 1.105 μg/ml and 0.1115 μg/ml. Like the stock solutions all calibration levels and validation samples were divided into aliquots anticipating the number needed for the validation experiments and stored frozen at −20°C until analysis. The calibration samples and validation samples were pretreated exactly the same way as the unknowns.

2.4. Sample pretreatment

Plasma

Plasma samples were thawed and vortex-mixed thoroughly for 15 s and then centrifuged for 10 min at 4000 rpm. An aliquot of 500 μl of the supernatant was transferred to a glass reaction tube and 500 μl of a 20 m*M* NaH_2PO_4 solution (pH 3.0) were added. After thorough mixing an aliquot of 100 μl of the solution containing the internal standard was added to the sample solution and vortex-mixed. No internal standard solution was used for blank samples. For liquid–liquid extraction of analyte and internal standard 6 ml of ethyl acetate were added and rotated for 10 min at approximately 50 rpm. A subsequent centrifugation for 10 min at 4000 rpm separated the organic phase from the plasma phase. Then the organic phase was transferred to another glass tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase and the tube was vortex-mixed for 15 s. Thereafter 200 μl *n*-hexane were added and the solution was vortex-mixed for a short time. Again the sample was centrifuged at 6000 rpm for 10 min and the organic phase layer was withdrawn by suction with a disposable glass pasteur-pipet connected to a vacuum pump. An aliquot of 100 μl of the remaining solution was transferred to an autosampler vial and 50 μl were injected onto the HPLC system.

Urine

Urine samples were thawed and thoroughly vortex-mixed and an aliquot of 100 μl of the sample was pipetted to a polystyrene-tube, 900 μl of purified water added and thoroughly mixed (this dilution step was not performed for calibration standards and validation samples, as these solutions

have already been weighed in diluted (1:10) urine during preparation of the standards). An aliquot of 200 μl of the solution was transferred into a 1.5-ml reaction tube and 50 μl of the internal standard solution were added. After vortex-mixing an aliquot of 100 μl was pipetted into an autosampler vial and 10 μl were injected onto the HPLC system.

2.5. Validation of the assay

Accuracy and precision of the assay as well as the linearity of the calibration curve were determined intra-day and inter-day on three different days. Recovery of the analyte and the internal standard following the sample clean-up procedures relative to aqueous solutions were determined at different concentration levels.

3. Results and discussion

3.1. Specificity, linearity and sensitivity

The specificity of the method was determined by screening blank plasma of six different healthy donors. Nitrofurantoin, an antibacterial active compound of Urospasmon, was investigated for interferences with sulfadiazine or the internal standard sulfamethazine. No other compounds were investigated as the assay was developed for use in studies with healthy volunteers with this compound. Representative chromatograms of a spiked plasma sample (0.238 μg/ml sulfadiazine) and plasma samples derived from a healthy volunteer prior to administration of sulfadiazine and 2 h after an oral dose of 150 mg sulfadiazine (0.0584 μg/ml sulfadiazine) are shown in Fig. 1 and Fig. 2. A urine sample collected 0–6 h after an oral dose of 150 mg sulfadiazine (50.17 μg/ml sulfadiazine) is shown in Fig. 3. Retention times for sulfadiazine were between 9.20 and 9.35 min, and for the internal standard sulfamethazine between 26 and 27 min. The internal standard was chosen for its similar chemical structure, therefore we accepted also the long total run time of 32 min. The use of a liquid–liquid extraction results in a much cleaner plasma extract compared to the chromatograms obtained with the previous method with simple protein precipitation. Sulfadiazine

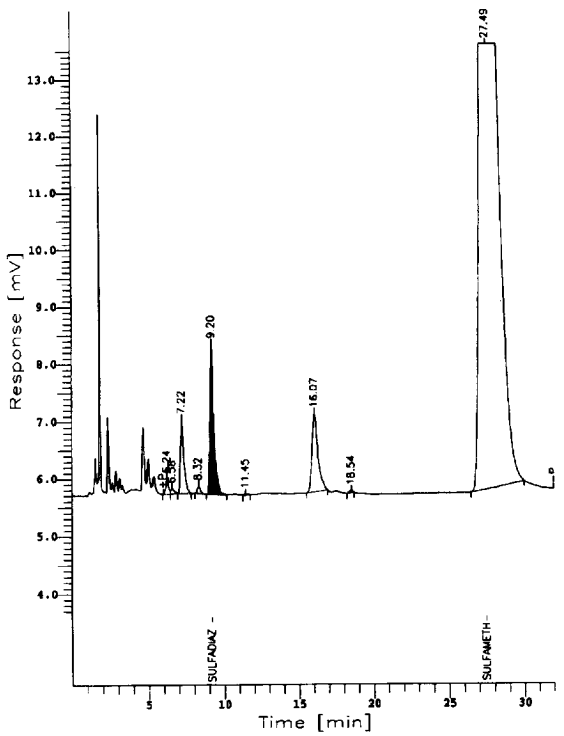

Fig. 1. Blank plasma spiked with 0.238 μg/ml sulfadazine and internal standard.

and the internal standard are completely resolved from each other as well as from any endogenous peaks in plasma and urine. Nitrofurantoin, the second compound in the pharmaceutical preparation to be tested, was not detected in the chromatographic system.

For evaluation of the calibration graph a weighted linear regression ($1/x$) was performed with nominal concentrations of calibration levels and measured peak-height ratios (peak-height analyte/peak-height internal standard). The slope and intercept of the ten-point regression graph were determined according to standard equations. Linearity of the assay could be shown over a concentration range of 0.0299–15.2 μg/ml. The coefficient of correlation (r^2) was above 0.9995 in each case. The intercept ranged between 1.6 and 11.9% of the response of the lowest level for the individual curves with a mean value of 6.78% (Table 1). The relative errors of the individual calibration points were between −8.25

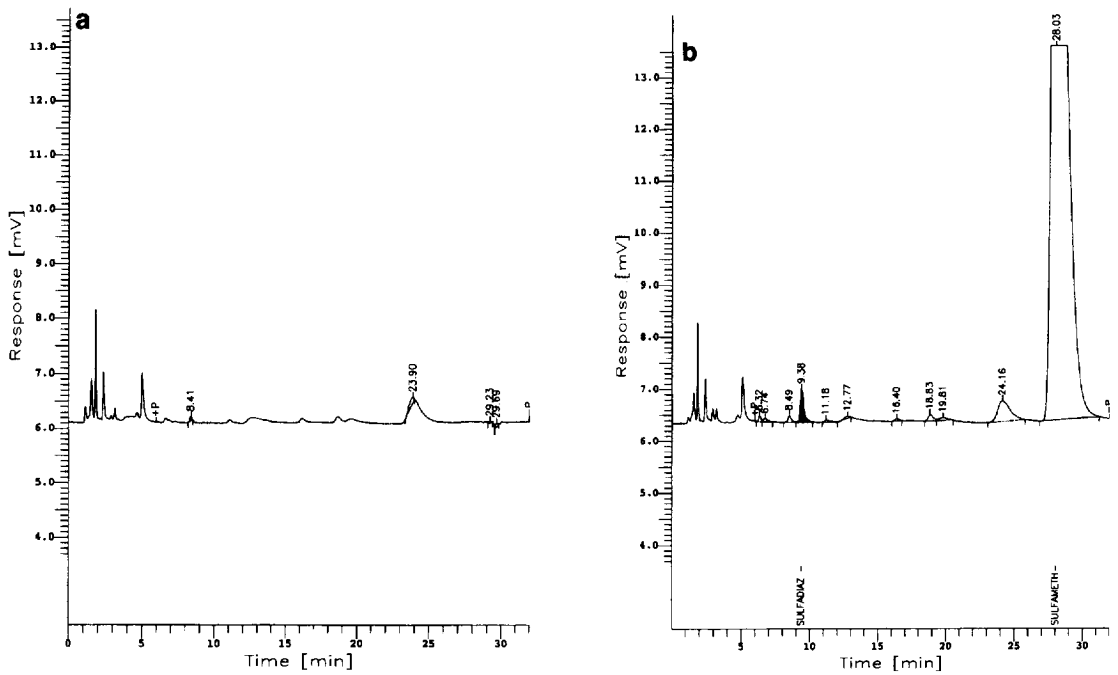

Fig. 2. (a) Blank plasma of a healthy volunteer prior to drug administration. (b) Plasma sample of a healthy volunteer collected 2 h after an oral dose of 150 mg sulfadiazine (sulfadiazine concentration 0.0584 μg/ml).

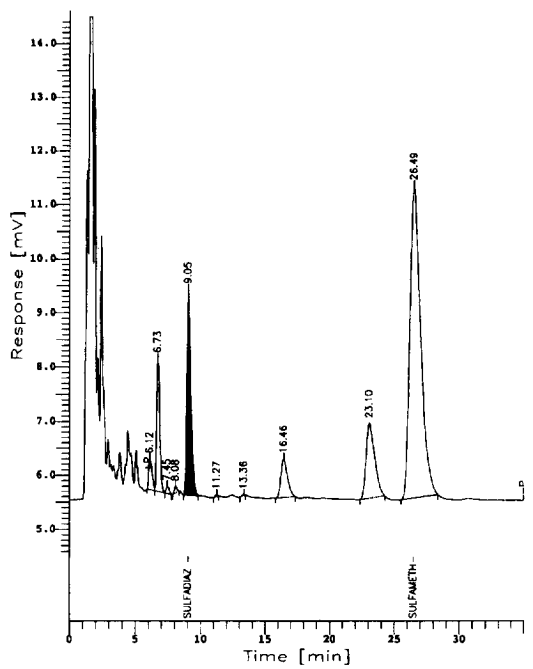

Fig. 3. Urine sample of a healthy volunteer collected 0–6 h after an oral dose of 150 mg sulfadiazine (sulfadiazine concentration 50.17 μg/ml).

and 7.33%. The mean accuracy of the different calibration levels was between 98.3 and 101%.

The lowest calibration level used in the validation calibration curve yielded a concentration of 0.0299 μg/ml sulfadiazine in plasma. This was set as limit of quantitation that can be measured with certain accuracy. The signal-to-noise level was about 15 at this calibration level. The individual values of the accuracy of this calibration level were between −8.25 and 7.33%. These values indicate that even a lower calibration level may be determined with sufficient precision and accuracy if demanded for pharmacokinetic reasons.

3.2. Precision and accuracy

The intra-day precision and accuracy was determined by analyzing five aliquots of each validation sample within one run. Means, standard deviations, coefficients of variation (%) and relative errors (%) were determined. The intra-day precision was good and consistent with values of 2.31% (n=3) for a concentration of 10.9 μg/ml sulfadiazine, 0.42%

Table 1
Calibration parameters of all runs

Parameter	Day 1 cal. curve 1	Day 1 cal. curve 2	Day 2 cal. curve 3	Day 2 cal. curve 4	Day 3 cal. curve 5	Day 3 cal. curve 6
Number of levels used	10	9	10	10	9	8
Slope	0.314	0.303	0.285	0.283	0.295	0.296
Intercept	−0.000726	0.000661	−0.000503	0.00109	0.000146	−0.000499
Coefficient of correlation, r^2	1.0000	0.9997	1.0000	0.9996	1.0000	0.9999
Response of lowest level	0.00933	0.00988	0.00731	0.00914	0.00902	0.00863
Intercept expressed as % of the response of the lowest level	7.8	6.7	6.9	11.9	1.6	5.8

(n=5) and 2.05% (n=5) for a concentration of 1.11 μg/ml and 0.111 μg/ml sulfadiazine, respectively (Table 2). Inter-day variation was determined by analyzing two calibration curves and two replicates of each validation sample on each of three different days. The respective values for the inter-day precision were very similar and support the robustness of the method. The mean value of the inter-day precision of the highest validation sample was 2.80%, the values for the medium and lower validation samples were 1.80% and 1.79% (Table 3). The accuracy was calculated as the relative error by compiling the measured concentrations with the nominal concentrations of sulfadiazine in validation samples. For the intra-day accuracy the individual deviations ranged from −3.3 to 1.2% for the high validation sample (10.9 μg/ml sulfadiazine), −0.4 to 0.6% for the medium validation sample (1.11 μg/ml), and −2.3 to 3.1% for the low validation sample (0.112 μg/ml). As already noted for the precision the inter-day accuracy was in a very similar range compared to the intra-day values. The individual values ranged from −6.1 to −0.5%, −2.5 to 2.9%, and −1.4 to 3.1% for the high, medium, and low validation samples, respectively.

3.3. Recovery

Recovery was measured by comparison of peak-heights of non-extracted standards in mobile phase versus extracted standards of spiked plasma. The recoveries of the analyte and the internal standard were determined separately. The recovery of sulfadiazine was determined as 74.9% (n=3) at 15.0 μg/ml, 70.6% (n=3) at 1.50 μg/ml and 68.4% (n=3) at 0.150 μg/ml. The recovery of the internal standard sulfamethazine was found to be 75.8% (n=3) at 10.0 μg/ml and 79.4% (n=3) at 1.00 μg/ml.

Table 2
Intra-day precision and relative error (R.E.) of validation samples (VS)

	VS 1		VS 2		VS 3	
	Conc. (μg/ml)	R.E. (%)	Conc. (μg/ml)	R.E. (%)	Conc. (μg/ml)	R.E. (%)
	10.9[a]		1.11[a]		0.112[a]	
	10.9	−0.55	1.11	0.60	0.111	−0.75
	10.6	−3.34	1.11	0.29	0.112	0.73
	11.1	1.21	1.11	0.22	0.111	−0.83
	–		1.10	−0.45	0.109	−2.35
	–		1.11	0.57	0.115	3.09
n	3		5		5	
Mean	10.8		1.11		0.111	
S.D.	0.3		0.00		0.002	
C.V. (%)	2.31		0.42		2.05	

[a] Nominal sulfadiazine concentration (μg/ml).
–=outlier not included in the calculation.

Table 3
Inter-day precision and relative error (R.E.) of validation samples (VS)

Analysed on day	VS 1		VS 2		VS 3	
	Conc. (μg/ml)	R.S. (%)	Conc. (μg/ml)	R.E. (%)	Conc. (μg/ml)	R.E. (%)
	10.9[a]		1.11[a]		0.112[a]	
1	10.9	−0.5	1.10	−0.6	0.110	−1.4
1	10.8	−1.0	1.08	−2.5	0.112	0.6
2	10.3	−6.1	1.10	−0.3	0.110	−1.0
2	10.6	−2.8	1.14	2.9	0.113	1.1
3	10.9	−0.5	1.11	0.6	0.111	−0.7
3	–		1.11	0.6	0.115	3.1
n	5		6		6	
Mean	10.7		1.11		0.112	
S.D.	0.3		0.02		0.002	
C.V. (%)	2.80		1.80		1.79	

[a] Nominal sulfadiazine concentration (μg/ml).
–=outlier not included in the calculation.

3.4. Urine

The HPLC method for measurement of sulfadiazine in human plasma was tested for the suitability to quantitate sulfadiazine in human urine. The only change of the assay conditions refers to the sample work-up: instead of deproteinizing by liquid–liquid extraction the urine samples were diluted with purified water. As the validation in plasma samples has proven the good performance of the method in general, the validation procedure for measurement of urine samples was limited to the fundamental assay parameters. The appropriate biological matrix, human drug-free (blank) urine, was collected from human donors. For preparation of calibration standards and quality controls the native urine was prediluted 1:10 with purified water. Stock solutions of sulfadiazine and sulfamethazine were prepared in purified water.

The normalized responses of the calibration standards showed a small variation of 4.09%. The calculated concentrations of calibration levels in all runs yielded an even lower coefficient of variation of 1.02%. These numbers indicate a good precision and confirm the corresponding results of the validation of the plasma assay. The calibration graph exhibited a very good linearity with a coefficient of correlation (r^2) of 1.0000 in the calibration range of 0.578–149 μg/ml. The relative errors of calibration standards ranged between −1.36% and +1.78%. The intercept was below 10% of the response of the lowest level. Since level no. 9 had a relative error of −0.22%, its nominal concentration of 0.578 μg/ml was accepted as quantitation limit. The analysis of the quality controls produced very small relative errors with −0.46% at 99.9 μg/ml sulfadiazine, +0.45% at 10.1 μg/ml sulfadiazine, and −2.09% at 1.01 μg/ml sulfadiazine.

4. Application

4.1. Plasma

The validated method was used to analyze 650 plasma samples from a pharmacokinetic study in healthy volunteers. Calibration levels and quality controls were identical to the calibration levels and validation samples used during the validation procedures. Samples were analyzed during a two-month period in 13 runs each including a complete calibration curve covering the whole concentration range of unknown samples and two sets of QCs. The values obtained for the calibration levels and the resulting calibration graphs were in a similar range as observed during validation. The coefficient of correlation (r^2) was above 0.9995 in each case. The intercept expressed relative to the response of the lowest calibration level ranged between 0.13 and 19.8% (36.8% in one case). The relative error of the

individual calibration points was between −12.21 and 9.45%. The inter-day precision of the quality controls was somewhat higher than observed during validation. The precision of the highest quality control (10.9 μg/ml sulfadiazine) was 3.68% (n=24), the medium quality control (1.11 μg/ml sulfadiazine) yielded a value of 2.03% (n=26) and the low quality control (0.112 μg/ml sulfadiazine) 10.4% (n=26).

4.2. Urine

More than 190 human urine samples were analyzed with the presented method. Samples were analyzed in a two-week period in five runs each including a complete calibration curve covering the whole concentration range of unknown samples and two sets of QCs. Similar to the plasma assay the quality data obtained during routine work confirmed the findings of the validation procedure. The coefficient of correlation (r^2) of the calibration graphs was greater than 0.9996 in each case. The intercept expressed as the percentage of the response of the lowest calibration level was between 0.16 and 19.77%. The precision of the quality controls was 2.17% (n=10) at 100 μg/ml sulfadiazine, 2.10% (n=10) at 10.1 mg/ml, and 4.97% (n=10) at 1.01 μg/ml.

5. Conclusions

This paper describes a sensitive, selective and reliable HPLC assay for sulfadiazine in human plasma and urine. The method includes a liquid–liquid extraction for plasma samples and a simple dilution step for urine samples. A linear quantitation range from 0.0299 to 15.2 μg/ml for plasma and 0.578 to 148.8 μg/ml for urine could be established. The method has been successfully used for measurement of samples derived from a human pharmacokinetic study after multiple dosing of Urospasmon. Plasma levels could be followed up until 48 h after last dosing. A daily throughput of 80–100 plasma samples by manual sample preparation and automated HPLC system is possible. The precision and accuracy as found during the validation procedure for plasma was in total confirmed during the routine analysis. The QCs, especially the low QC, were found to be somewhat more imprecise, whereas the values of the respective quality controls for the urine assay were quite the same as during the validation procedures. This may reflect the more complex nature of sample preparation of plasma samples.

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