ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 1373–1382

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

New specific and sensitive HPLC-assays for ethacrynic acid and its main metabolite — the cysteine conjugate — in biological material ☆

Barbara Voith *, Hildegard Spahn-Langguth [1], Ernst Mutschler

Department of Pharmacology, J.W. Goethe-University, Biocenter Niederursel, Marie-Curie-Str. 9, Bldg. N 260, D-60439 Frankfurt, Germany

Received for review 3 April 1995

Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method was developed and validated for the determination of the loop diuretic ethacrynic acid and its potentially active main metabolite, the ethacrynic acid–cysteine conjugate, in biological material. Simple and rapid sample preparation procedures were established using solid-phase extraction for the parent drug and direct injection after one washing step for the metabolite. HPLC separation was performed on a Spherisorb ODS II (3 μm) analytical column using isocratic elution with different mixtures of mobile phases (phosphoric acid–methanol–acetonitrile–tetrahydrofuran or triethylamine buffer–methanol, respectively). The analytes were detected by measuring the UV absorption of the eluate at 275 nm. Stability studies revealed that considerable amounts of ethacrynic acid may be released from the cysteine conjugate unless the urine samples are pH stabilized (pH 3–4). The assay provided high sensitivity with limits of quantification of 20 ng ml^{-1} for ethacrynic acid in plasma and urine, and 240 ng ml^{-1} for the cysteine conjugate in urine. All validation parameters were within the required limits. For the presented assays, the applicability to pharmacokinetic studies and routine analyses was proved.

Keywords: High-performance liquid chromatography; Ethacrynic acid; Etacrynic acid; Cysteine conjugate; Solid-phase extraction

1. Introduction

Ethacrynic acid ((2,3-dichloro-4-(2-methylenebutyryl)phenoxy)-acetic acid) (Fig. 1(a)) is an unsaturated ketone derivative of phenoxyacetic acid and is chemically different from all other known diuretic agents. It belongs to the group of loop diuretics and is characterized by a rapid onset and a short duration of action [1]. In various in vitro studies, it was demonstrated that its mode of action seems to be independent of a blockade of the $Na^+/K^+/2Cl^-$ carrier [2,3].

Although parts of the metabolic pattern still have to be established, the main biotransformation product in dogs and rats is known to be a conjugate with L-cysteine (Fig. 1(b)) [1,4]. For this metabolite, a diuretic activity has already been postulated from in vitro studies using different test models for diuretic activity [3,5].

In addition to the classical indications for loop diuretics, the drug has gained new rele-

☆ Presented in part as short communications at the following symposia: Joint meeting of the German Pharmacological Society and the French Pharmacological Society, Lille, France, October 1993; Annual symposium of the German Pharmaceutical Society, Saarbrücken, Germany, September 1993.

* Corresponding author.

[1] Present address: Faculty of Pharmacy, Martin-Luther-University, Weinbergweg 15, D-06120 Halle-Wittenberg, Germany.

SSDI 0731-7085(95)01558-2

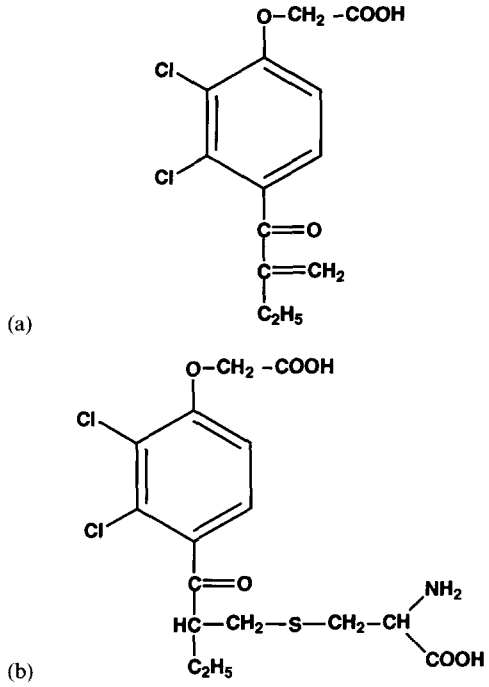


Fig. 1. Chemical structures of (a) ethacrynic acid and (b) ethacrynic acid–cysteine conjugate.

vance during the last years because of its usefulness in the treatment of a particular type of tumor presenting a drug-resistant phenotype through elevated glutathione *S*-transferase (GST) levels, a glutathione (GSH) dependent isoenzyme family. Ethacrynic acid seems to be able to inhibit GST-mediated conjugation of alkylating cancer drugs with GSH [6].

A variety of methods for the quantification of ethacrynic acid have been reported. Most of them are only suitable for determination of the drug and its degradation products in aqueous solution [7–9]. Gas chromatographic determination of the drug in biological material requires either derivatization prior to analysis [10] or electron-capture detection [11]. The only reported HPLC method suitable for the analysis of biological material until now [12] lacks sensitivity for determination of the low plasma concentrations after oral application of the therapeutic dosage of 50 mg. So far, no method has been reported for the determination of the cysteine conjugate in biological material. However, in a very recent publication [13] ethacrynic acid was used as a coupling reagent in an assay for L-cysteine because of its high reactivity at the α-keto-double bond.

It was the aim of the present study to develop a rapid, specific and sensitive HPLC-assay for ethacrynic acid in plasma and urine, and its major metabolite in urine, in order to be able to develop a kinetic/dynamic model for the renal effects observed following ethacrynic acid dosage. In the course of the studies, it was hypothesized that, potentially, an acyl glucuronide may be formed as an additional metabolite, which may be unstable at physiological pH [14]. Hence, plasma and urine samples of volunteers were acidified in order to prevent acyl migration and hydrolysis, if a glucuronide was present [14].

2. Experimental

2.1. Chemicals

All chemicals were supplied by E. Merck (Darmstadt, Germany) unless stated otherwise. Acetonitrile, methanol and tetrahydrofuran were of HPLC grade, and all other solvents and reagents were of analytical grade. Ethacrynic acid (EA) was kindly provided by Merck, Sharp and Dohme (München, Germany). The internal standards, 4-(2,4-dichlorophenoxy)-butyric acid (DCBA) and diclofenac, as well as triethylamine, were purchased from Sigma GmbH (Deisenhofen, Germany). The ethacrynic acid–cysteine conjugate (EACYS) and the glutathione conjugate (EAGSH), used as internal standard for EACYS, were synthesized according to Ref. [15]. Blank pool plasma was obtained from the Hessischer Blutspendedienst (Frankfurt, Germany).

2.2. Standard solutions

Stock solutions of EA, diclofenac and DCBA were prepared using methanol as solvent. (EA, 1 μg ml^{-1}, 10 μg ml^{-1} and 100 μg ml^{-1}, internal standards, 3 μg ml^{-1} for diclofenac and 7 μg ml^{-1} for DCBA.) Stock solutions for EACYS and EAGSH were prepared using bidistilled water yielding a concentration of 1.5 mg ml^{-1} for EAGSH, and 12 μg ml^{-1}, 120 μg ml^{-1} and 1.2 mg ml^{-1} for EACYS. All stock solutions were stored at 4 °C for several weeks without any detectable degradation.

2.3. Equipment

The HPLC system consisted of a Knauer pump 64 (Knauer, Berlin, Germany), a Spectra-

Physics autosampler SP 8880 with a 200 µl filling loop (Thermo Separation Products, Darmstadt, Germany) and a Jasco 960 UV detector (Jasco, Groß-Umstadt, Germany). Data analysis was performed using a Spectra-Physics Chromjet integrator connected to Spectra-Physics WINer 386 software (Thermo Separation Products, Darmstadt, Germany). Solid-phase extraction was performed with a Varian Vac Elut 224 (ICT, Bad Homburg, Germany). For centrifugation, a Heraeus Minifuge GL (Heraeus Christ, Hanau, Germany) was used. Evaporation of organic solvents was conducted using a Speed Vac Concentrator 200 H (Bachhofer, Reutlingen, Germany).

2.4. Sample preparation

Ethacrynic acid

Solid-phase extraction was conducted prior to HPLC using Varian Bond Elut®-C_{18} phases (part. no.: 1210-2025, ICT, Eschborn, Germany). The cartridge was activated with two column volumes of methanol and one column volume of bidistilled water. 1 ml of acidified plasma sample or 500 µl of acidified urine (for acidification procedure see Section 2.8) was then added after mixing the sample thoroughly with 100 µl 1 M hydrochloric acid for further acidification and 70 µl of internal standard solution (diclofenac for plasma samples and DCBA for urine samples). The adsorbent was washed with one column volume of bidistilled water and subsequently with 4 ml of an ethanol–water mixture (9:1, v/v). Finally, the analytes were eluted with 800 µl of pure ethanol. The ethanol fraction was evaporated to dryness, the residue dissolved in 100 µl mobile phase and an aliquot of 70 µl injected onto the HPLC column. The total recovery of EA was $60 \pm 3.4\%$ and $62 \pm 4.1\%$ ($n = 6$, 100 and 500 ng ml^{-1}) for extraction from plasma, and $58 \pm 2.8\%$ and $61 \pm 3.2\%$, respectively, for urine compared to unextracted solution.

Cysteine conjugate

Owing to the high hydrophilicity of this compound, extraction from biological material was not feasible. The urine samples were mixed with 30 µl of internal standard solution (EAGSH) and washed with a mixture of dichloromethane–2-propanol (60:40, v/v) by shaking for 30 min and centrifugation (15 min, 0 °C, 4000 rpm). The organic layer was discarded and the aqueous layer directly injected onto the column. No EACYS was detectable in the organic layer. The recovery for this compound following pre-extraction was 100%.

2.5. Chromatographic conditions

Plasma analysis

Ethacrynic acid. For the determination of EA, a Spherisorb ODS II column, 125 × 4.6 mm i.d., with a 10 × 4.6 mm guard column filled with the same material (both Bischoff, Leonberg, Germany) was used as the stationary phase, with a mixture of phosphoric acid 0.2%, acetonitrile, methanol and tetrahydrofuran (50:32:13:1.5, v/v/v/v) as the mobile phase. The guard column was freshly refilled every week. At ambient temperature a flow-rate of 0.9 ml min^{-1} resulted in an average pressure of 19 MPa. The UV-absorbance of the eluate was measured at 275 nm. Under these conditions, the retention times for EA and the internal standard diclofenac were on average 19 and 37 min, respectively.

Urine analysis

Ethacrynic acid. Because of interfering urine constituents, the chromatographic conditions for the determination had to be slightly modified when compared to the plasma assay. The internal standard diclofenac was substituted by DCBA, and the mobile phase was modified (phosphoric acid 0.2% – acetonitrile – methanol – tetrahydrofuran, 55:32:13:2, v/v/v/v). A flow-rate of 0.8 ml min^{-1} yielded average retention times of 30 and 32 min for EA and DCBA, respectively. The mean pressure was in the same range as for plasma analysis.

Cysteine conjugate. For the determination of EACYS, the same stationary phase was used, but with a mixture of 0.1 M triethylamine buffer (adjusted to pH 3 with phosphoric acid) and methanol (28:30, v/v) as the mobile phase. Analysis was conducted at ambient temperature and at a flow-rate of 0.8 ml min^{-1}, resulting in an average pressure of 20 MPa. The mean retention times for EACYS and EAGSH, which was used as internal standard, were 13 and 17 min, respectively.

2.6. Assay validation

Calibration data were generated by spiking blank plasma and urine samples with EA and

EACYS, respectively, yielding concentrations of 20–3000 ng ml^{-1} for EA in plasma and urine, and 240 ng ml^{-1}–12 μg ml^{-1} for EACYS in urine. Calibration curves were generated by plotting the ratios of the peak areas (EA/IS; EACYS/IS) versus concentration and calculating the linear regression ($y = a + bx$; weighting $y = 1$).

Inter-day precision and accuracy were determined using eight samples of at least three concentrations analyzed on eight different days ($n = 8$). Intra-day accuracy was assessed using eight samples of three different concentrations, one at the limit of quantification, and one at a medium and one at a high concentration. These samples were prepared and analyzed on the same day.

Precision and accuracy were characterized by relative standard deviation (RSD) and by the difference between the nominal and found concentration, respectively, expressed as a percentage (RE%).

2.7. Stability studies

Owing to the fact that formation of the cysteine conjugate was found to be a reversible process [15], stability studies in pooled human urine of physiological pH (6.5) as well as in stabilized samples of pH 3 were conducted. Therefore, spiked urine samples containing 5 μg ml^{-1} EACYS, one set at physiological pH (pH 6.5), one set at pH 3 with phosphoric acid (5 μl phosphoric acid 85% ml^{-1} urine), were incubated at 37 °C for 7 h. Degradation of EACYS was determined by measuring the increasing concentration of free EA after 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 7 h. At the end of the incubation time, internal standard was added and sample preparation was performed as described above.

In the same manner, the stability of EAGSH, the internal standard for urine analysis, was tested at physiological pH and after acidification.

The stability of EAC as well as EAGSH during the analysis period was proved by analysis of quality control samples distributed over the whole run and comparison of the concentration found at the beginning and at the end of each run, respectively.

2.8. Biological samples

The applicability of the presented method was shown in a pilot study conducted with three healthy volunteers receiving 50 mg of EA either as i.v. bolus or orally as a tablet. Plasma and urine samples were obtained at appropriate time intervals. All samples were pH- and temperature-stabilized immediately after collection by adding 30 μl phosphoric acid (86%) to a 4 ml aliquot of plasma (50 μl phosphoric acid to 10 ml urine) in order to adjust the samples to pH to 3–4, and stored frozen at −22 °C until analysis.

The pilot study was followed by a clinical trial with 12 healthy volunteers, in order to obtain detailed knowledge concerning the pharmacokinetic behavior of EA and its hypothesized main metabolite, EACYS.

3. Results and discussion

As a result of the present work, a reliable assay for EA and its main metabolite is available, which also proved its usefulness in clinical trials. Representative chromatograms obtained from one volunteer are shown in Fig. 2, compared to blank plasma or urine, respectively. These chromatograms indicate good separation of the analytes as well as the internal standards from endogenous compounds. As demonstrated in Fig. 2(b), for plasma analysis a variety of interfering peaks with retention times from 22 up to 38 min were detected. Hence, an appropriate internal standard had to be selected to fit into a "window", i.e. which was clearly separated from these peaks. Diclofenac does not only fulfill these chromatographic requirements, but also yielded similar extraction rates when compared with EAC, thus leading to a significant increase in the precision of analysis (e.g. RSD of 21% without and 4.8% with internal standard at a concentration of 500 ng ml^{-1} EA). Since the respective interfering peaks were not present in urine extract, DCBA, a compound with a significantly shorter retention time, was suitable as internal standard for urine as opposed to plasma. This modification reduced the total runtime from 50 min for plasma to 35 min for urine samples. No interferences between the analytes and possible further (known) metabolites were detected under the present conditions, which was confirmed by studies with diode-array UV detection at higher EA levels. The specificity of the meth-

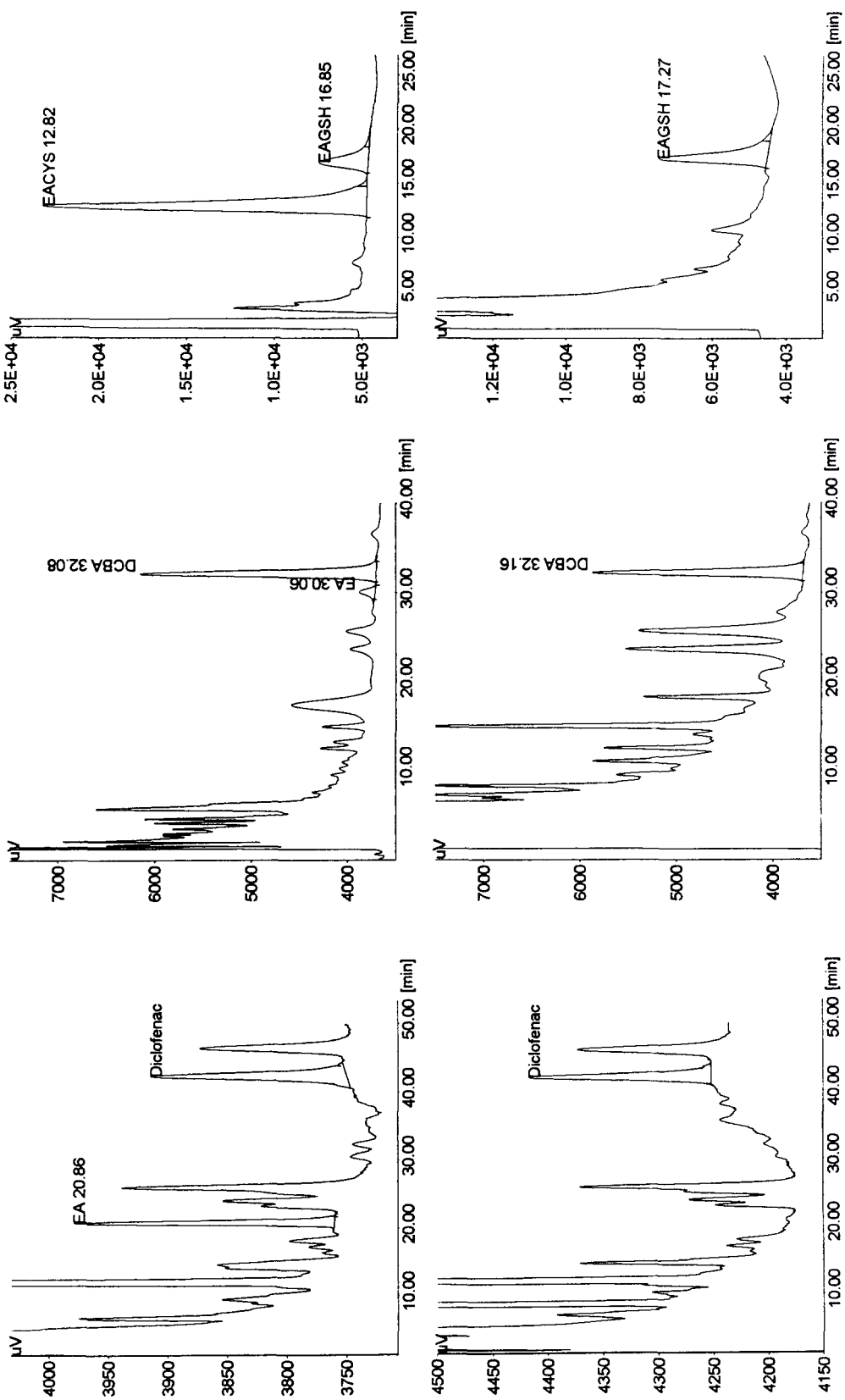


Fig. 2. Chromatographic separation following extraction and chromatography on a Spherisorb ODS column: biological samples compared with blank matrix: (a) EA in plasma, 0.5 h after i.v. administration (478.5 ng ml^{-1}); (b) EA in urine, 2 h after i.v. administration (57.8 ng ml^{-1}); (c) EACYS in urine, 1 h after i.v. administration (11.6 μg ml^{-1}).

Table 1
Linearity of calibration curves at low (a) and high (b) concentrations, and over the whole range (c) ($n = 6$)
(a) Linearity at low concentrations

	EA in plasma (20–500 ng ml^{-1})			EA in urine (20–500 ng ml^{-1})			EAC in urine (240 ng ml^{-1}–3.6 μg ml^{-1})		
	Correlation coefficient	Intercept	Slope	Correlation coefficient	Intercept	Slope	Correlation coefficient	Intercept	Slope
Mean value	0.99932	0.0226	0.00166	0.9985	−0.00236	0.000946	0.994	−0.0794	0.2587
SD	0.00571	0.0103	0.00010	0.0149	0.00106	0.000066	0.00525	0.0686	0.0205
RSD (%)	0.057	45.6	6.2	0.15	44.9	6.9	0.53	86.4	7.9

(b) Linearity at high concentrations

	EA in plasma (500–3000 ng ml^{-1})			EA in urine (500–3000 ng ml^{-1})			EAC in urine (3.6–12 ng ml^{-1})		
	Correlation coefficient	Intercept	Slope	Correlation coefficient	Intercept	Slope	Correlation coefficient	Intercept	Slope
Mean value	0.9997	0.0239	0.00159	0.9986	0.0255	0.0009225	0.97	−0.0585	0.2509
SD	0.00025	0.024	0.00016	0.001613	0.05511	0.0000584	0.0113	0.08479	0.01938
RSD (%)	0.025	100.3	10	0.16	216	6.3	0.11	144.9	7.7

(c) Linearity over the whole range

	EA in plasma (20–3000 ng ml^{-1})			EA in urine (50–3000 ng ml^{-1})			EAC in urine (240 ng ml^{-1}–12 μg ml^{-1})		
	Correlation coefficient	Intercept	Slope	Correlation coefficient	Intercept	Slope	Correlation coefficient	Intercept	Slope
Mean value	0.9997	0.0155	0.00158	0.99950	0.007694	0.0009347	0.9974	−0.1022	0.2533
SD	0.000265	0.0117	0.000162	0.00052	0.006957	0.000060	0.00112	0.0489	0.0225
RSD (%)	0.026	75.5	10.3	0.052	90.4	6.4	0.01	47.8	8.9

ods was tested using at least six different blank matrices, none of them presenting a detectable peak at the crucial retention times. In particular, the occurrence of a detectable peak at the retention time of EAGSH, the ethacrynic acid-conjugate which was used as internal standard for EACYS, was checked in the blank samples as well as in the biological samples obtained after administration of EA.

With a previously reported HPLC method using liquid–liquid extraction from plasma [12], separation of EA from interfering endogenous compounds was not accomplished, especially when low concentrations of EA were to be detected. Therefore, a new solid-phase extraction method was developed for sample preparation. The employed stationary phase used for this solid–liquid extraction was found to be appropriate owing to good adsorption of the analyte from aqueous solutions and quantitative desorption when using ethanol as eluent. For washing phases consisting of ethanol–water (1:9, v/v) the best results were obtained with respect to the elution of interfering compounds without any detectable loss of analytes. Thus, this method did not only provide good chromatographic separations from endogenous substances, but also led to a remarkable reduction in the use of organic solvents, which is considered an advantage from toxicological, environmental and financial points of view.

The calibration curves obtained with a spiked matrix showed good linearity in the concentration ranges mentioned above, i.e. in those relevant for clinical studies with therapeutic doses. Average correlation coefficients of six calibration curves analyzed on three subsequent days were >0.999. Recalculation of the standard concentrations resulted in deviations of $<10\%$ for all concentrations. By performing regression analysis with the upper and lower sections of the calibration curves, it was shown that the slope of the curves did

Table 2
Inter-day variability ($n = 8$)
(a) EA in plasma

Nominal concentration (ng ml^{-1})	Concentration found (ng ml^{-1})		RSD (%)	RE (%)
	Mean	SD		
100	91	8.5	9.3	−9
200	201.8	11.1	5.5	0.9
500	515.7	22.1	4.3	3.1

(b) EA in urine

Nominal concentration (ng ml^{-1})	Concentration found (ng ml^{-1})		RSD (%)	RE (%)
	Mean	SD		
100	109.6	7.8	6.5	9.6
500	534.5	50.4	9.4	6.9
1000	1077.2	77.7	7.7	7.7

(c) EAC in urine

Nominal concentration (ng ml^{-1})	Concentration found (ng ml^{-1})		RSD (%)	RE (%)
	Mean	SD		
1.4	1.38	0.011	0.8	−1.2
3.6	3.49	0.019	5.4	−4
4.2	4.05	0.032	7.9	−4

not deviate markedly over the entire concentration range (Table 1).

The results of inter-day and intra-day variation are shown in Tables 2 and 3. RSDs were below 10% for all concentrations for intra-day as well as for inter-day precision. By calculating the accuracy (RE%) the reliability of the method has also been proved. Under the conditions described, the limits of quantification, defined as the lowest concentration with RSD < 20%, were 20 ng ml^{-1} for EA in plasma and urine, and 240 ng ml^{-1} for EACYS in urine. The value for the latter substance was markedly higher owing to the fact that no concentration of the sample was performed. However, since the urinary concentrations of the metabolite were found to be 10–20 times higher than that of the parent drug, this limit of quantification is more than sufficient for clinical trials.

The stability of the compounds in biological matrices was investigated and was found to be of crucial importance. Control samples (adjusted to pH 3) frozen at −22 °C for up to three months showed neither significant loss in concentration of EA nor considerable cleavage of the cysteine conjugate when compared to freshly prepared samples. In addition, the stability of EACYS was tested in urine samples of physiological pH compared to stabilized samples of pH 3 at 37 °C. It was shown that degradation of EACYS was rather rapid at physiological pH (6.5), presenting a half-life of 1.2 h. During the observation period of 7 h, about 80% of the EACYS was cleaved to give free EA. In contrast, the stabilized samples did not provide a remarkable increase in concentration of free EA during the 7 h (Fig. 3). For EAGSH, used as internal standard for EACYS, stability was proved irrespective of pH.

The acidification of plasma and urine samples, initially performed in order to stabilize a potential acyl glucuronide — as already described in the reviews by Faed [16] and Spahn-Langguth and Benet [14] — turned out to be an important prerequisite for the assay of the cysteine conjugate as well, as clearly demonstrated by the stability studies performed during the validation process. Analysis of urine samples without this acidification would lead to an overestimation of the amount of parent drug excreted into urine, because degradation of the adduct will occur not only during thaw-

Table 3
Intra-day variability ($n = 8$)
(a) EA in plasma

Nominal concentration (ng ml^{-1})	Concentration found (ng ml^{-1})		RSD (%)	RE (%)
	Mean	SD		
20	19.1	0.876	4.6	−4.5
500	512	24.6	4.8	2.4
1000	1003.1	39.1	3.9	0.3

(b) EA in urine

Nominal concentration (ng ml^{-1})	Concentration found (ng ml^{-1})		RSD (%)	RE (%)
	Mean	SD		
20	18.16	1.7	9.4	−5
100	95	4.37	4.6	−9.2
500	510.2	38.7	7.6	2.1

(c) EAC in urine

Nominal concentration (ng ml^{-1})	Concentration found (ng ml^{-1})		RSD (%)	RE (%)
	Mean	SD		
0.24	0.23	0.0248	10.8	−4
2.4	2.2	0.128	5.8	−8
6.	6.2	0.273	4.4	3.3

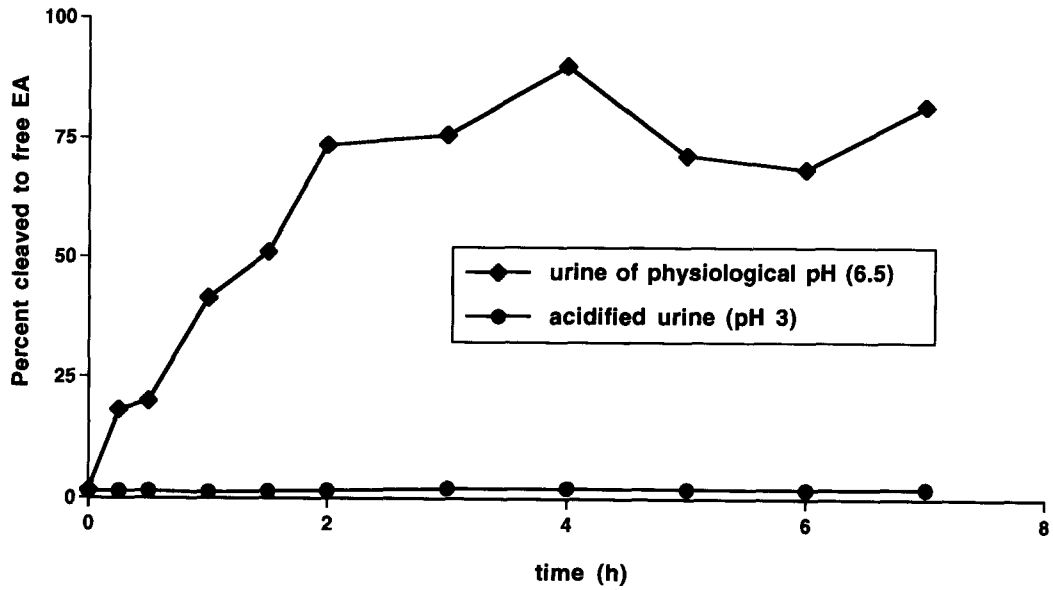
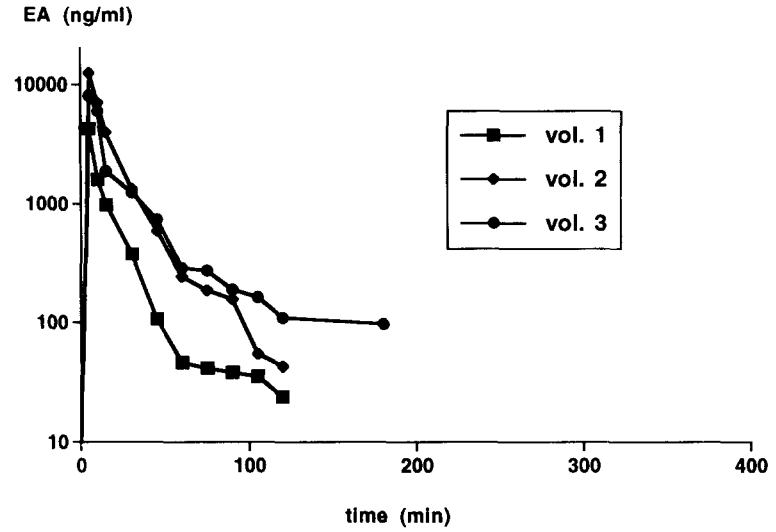


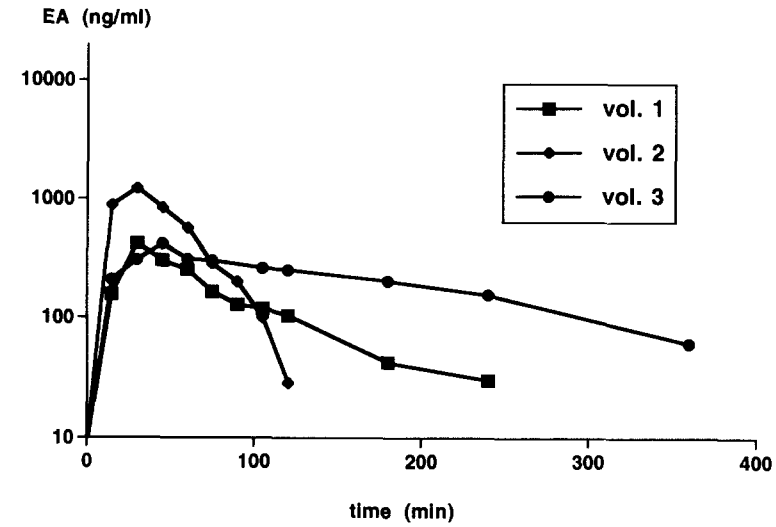
Fig. 3. Regeneration of EA from its cysteine conjugate at different pH values: percentage cleaved to give free EA during incubation of urine of physiological pH 6.5 (◆) and acidified urine of pH 3 (●) at 37 °C.

ing of the frozen sample, but also in the time period between sampling and freezing.

As mentioned above, the applicability of the described method has been proved in a clinical trial with three healthy male volunteers. Owing to the high sensitivity of the presented method compared to previously described assays [12], it was possible to determine the plasma concentration–time profile after i.v. as well as after p.o. application of 50 mg EA as shown in Fig.



(a)



(b)

Fig. 4. Plasma concentration–time profiles obtained during the pilot study after administration of 50 mg EA: (a) after i.v. administration; (b) after p.o. administration.

4. It is clearly demonstrated that the low limit of detection allows a reliable evaluation of the terminal half-life for both application forms.

4. Conclusions

The described methods were found to be reliable and sensitive, and thus are highly qualified for the routine analysis necessary in pharmacokinetic studies. The method described for EA provided similar or even better sensitivity than obtained with the reported GC-methods, without any necessity for derivatization or complex instrumentation. In addition, a bioanalytical assay for the main metabolite was established for the first time. The acidification performed with the biological samples proved to be a crucial step in the determination of correct concentrations of parent drug and metabolite. This was of particular interest for further investigation of their diuretic activity, since a significant fraction of the cysteine conjugate may be rapidly converted to free EA at physiological pH values, unless the sample is kept at a very low temperature.

Acknowledgments

Parts of the equipment used for this study were supported by the Dr. Robert-Pfleger-Stiftung, Bamberg, Germany, and the Fond der Chemischen Industrie, Germany.

References

[1] K.H. Beyer, J.E. Baer, I.K. Michaelson and H.F. Russo, J. Pharmacol. Exp. Ther., 147 (1965) 1–22.

[2] E. Schlatter, R. Greger and C. Weidtke, Pflügers Arch., 396 (1983) 210–217.

[3] H.C. Palfrey and S. Leung, Am. J. Physiol., 264 (1993) C1270–C1277.

[4] C.D. Klaassen and T.J. Fitzgerald, J. Pharmacol. Exp. Ther., 191 (1974) 548–556.

[5] M. Burg and N. Green, Kidney Int., 4 (1973) 301–308.

[6] P.J. Ciaccio, K.D. Tew and F.P. LaCreta, Biochem. Pharmacol., 42 (1991) 1504–1507.

[7] E.M. Cohen, J. Pharm. Sci., 60 (1971) 1702–1704.

[8] V. Cavrini, D. Bonazzi, A.M. Di Pietra and R. Gatti, Analyst, 114 (1989) 1307–1310.

[9] R.J. Yarwood, W.D. Moore and J.H. Collett, J. Pharm. Sci., 74 (1985) 220–223.

[10] W. Stüber, E. Mutschler and D. Steinbach, J. Chromatogr. Biomed. Appl., 227 (1982) 193–198.

[11] W.R. Sullivan and K.E. Fox, J. Chromatogr. Biomed. Appl., 425 (1988) 396–405.

[12] F.P. LaCreta, J.M. Brennan, P.W. Tinsley and P.J. O'Dwyer, J. Chromatogr. Biomed. Appl., 571 (1991) 271–276.

[13] A.M. DiPietra, R. Gotti, D. Bonazzi, V. Andrisano and V. Cavrini, J. Pharm. Biomed. Anal., 12 (1994) 91–98.

[14] H. Spahn-Langguth and L.Z. Benet, Drug Metab. Rev., 24 (1992) 5–48.

[15] D.A. Koechel and E.J. Cafruny, J. Med. Chem., 16 (1973) 1147–1152.

[16] E.M. Faed, Drug Metab. Rev., 15 (1984) 1213–1249.